

BIRCH, STEWART, KOLASCH & BIRCH, LLP

INTELLECTUAL PROPERTY LAW  
8110 GATEHOUSE ROAD  
SUITE 500 EAST  
FALLS CHURCH, VA 22042  
U S A

(703) 205-8000

FAX: (703) 205-8050

(703) 698-8590 (G IV)

e-mail: mailroom@bskb.com

web: http://www.bskb.com

GARY D. YACURA  
THOMAS S. AUCHTERLO  
MICHAEL R. CAMMARATA  
JAMES T. ELLER, JR.  
SCOTT L. LOWE  
JOSEPH H. KIM, Ph.D.\*  
RICHARD S. MYERS, JR.\*  
MARY ANN CAPRIA  
MICHAEL J. CORNELISON\*  
MARK J. NUEL, Ph.D.  
ROBERT V. RACUNAS  
DARIN E. BARTHOLOMEW\*  
D. RICHARD ANDERSON  
PAUL C. LEWIS  
JERRY W. HOGGE

REG. PATENT AGENTS:  
FREDERICK R. HANDREN  
ANDREW J. TELESZ, JR.  
MARYANNE LIOTTA, Ph.D.  
MAKI HATSUMI  
STEVEN P. WIGMORE  
ESTHER H. CHIN  
MIKE S. RYU  
W. KARL RENNER  
CRAIG A. McROBBIE  
GARTH M. DAHLEN, Ph.D.  
LAURA C. LUTZ  
ROBERT E. GOOZNER, Ph.D.

04/09/99  
09/289180  
PTO



TERRELL C. BIRCH  
RAYMOND C. STEWART  
JOSEPH A. KOLASCH  
JAMES M. SLATTERY  
BERNARD L. SWEENEY\*  
MICHAEL K. MUTTER  
CHARLES GORENSTEIN  
GERALD M. MURPHY, JR.  
LEONARD R. SVENSSON  
TERRY L. CLARK  
ANDREW D. MEIKLE  
DARC S. WEINER  
JOE MCKINNEY MUNCY  
ROBERT J. KENNEY  
C. JOSEPH FARACI  
DONALD J. DALEY  
JOHN W. BAILEY  
JOHN A. CASTELLANO, III

SENIOR COUNSEL:  
ANTHONY L. BIRCH

OF COUNSEL:  
HERBERT M. BIRCH (1905-1996)  
ELLIOT A. GOLDBERG\*  
WILLIAM L. GATES\*  
EDWARD H. VALANCE  
RUPERT J. BRADY (RET.)\*

\*ADMITTED TO A BAR OTHER THAN VA

Date: April 9, 1999

Docket No.: 2185-0324P-SP

Assistant Commissioner for Patents  
Box PATENT APPLICATION  
Washington, D.C. 20231

Sir:

As authorized by the inventor(s), transmitted herewith for filing  
is a patent application applied for on behalf of the inventor(s)  
according to the provisions of 37 CFR 1.41(c).

Inventor(s): SHIMOKAWATOKO, Yasutaka  
NISHIO, Shoichi

For: A METHOD FOR EVALUATING THE ABILITY OF A COMPOUND TO  
INHIBIT THE PROTOPORPHYRINOGEN OXIDASE ACTIVITY

Enclosed are:

- ☒ X A specification consisting of 74 pages
- ☒ X 3 sheet(s) of Formal drawings
- ☒ X Certified copy of Priority Document(s)
- ☐ X Executed Declaration in accordance with 37 CFR 1.64 will follow
- ☐ A verified statement to establish small entity status under 37  
CFR 1.9 and 37 CFR 1.27
- ☐ Preliminary Amendment
- ☒ X Information Sheet
- ☐ Information Disclosure Statement, PTO-1449 with reference(s)

Other \_\_\_\_\_

The filing fee has been calculated as shown below:

LARGE ENTITY				SMALL ENTITY	
FOR	NO. FILED	NO. EXTRA	RATE FEE		RATE FEE
BASIC FEE	***** ***** *****	***** ***** *****	***** ***** \$760.00 *****	or	***** ***** \$380.00 *****
TOTAL CLAIMS	65 - 20 =	45	x18 =\$ 810.00	or	x 9 = \$ 0.00
INDEPENDENT	14 - 3 =	11	x78 =\$ 858.00	or	x 39 = \$ 0.00
MULTIPLE DEPENDENT CLAIM PRESENTED <u>yes</u>			+260 = \$260.00	or	+130 = \$ 0.00
TOTAL \$2,688.00				TOTAL \$ 0.00	

X The application transmitted herewith is filed in accordance with 37 CFR 1.41(c). The undersigned has been authorized by the inventor(s) to file the present application. The original duly executed patent application together with the surcharge will be forwarded in due course.

X A check in the amount of \$ 2688.00 to cover the filing fee and recording fee (if applicable) is enclosed.

\_\_\_\_\_ The Government Filing Fee will be paid at the time of completion of the filing requirement.

\_\_\_\_\_ Please charge Deposit Account No. 02-2448 in the amount of \$\_\_\_\_\_. A triplicate copy of this transmittal form is enclosed.

X Send Correspondence to: BIRCH, STEWART, KOLASCH & BIRCH, LLP  
P. O. Box 747  
Falls Church, Virginia 22040-0747

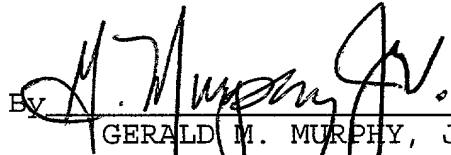
554040 "03T53250

— No fee is enclosed.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. 1.16 or under 37 C.F.R. 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By  \_\_\_\_\_  
GERALD M. MURPHY, JR.

Reg. No. 28,977

P. O. Box 747

Falls Church, Virginia 22040-0747

(703) 205-8000  
GMM/sas

02-2448-0324P-SP

## SPECIFICATION

A method for evaluating the ability of a compound to inhibit the protoporphyrinogen oxidase activity

### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention

The present invention relates to a method for evaluating the ability of a compound to inhibit the protoporphyrinogen oxidase activity.

#### 2. Description of the Related Art

Plants, animals and microorganisms possess the porphyrin biosynthetic system starting with 5-aminolevulinic acid and produce protoporphyrin which is a precursor for the heme biosynthesis and the like. An enzyme which catalyses a reaction oxidizing protoporphyrinogen at the final stage of the porphyrin biosynthesis to produce protoporphyrin is protoporphyrinogen oxidase (protoporphyrinogen IX oxidase; EC 1.3.3.4)(hereinafter referred to as "PPO")(R. J. Porra and J. E. Falk, (1964) Biochem. J., Vol.90, pp.69-75).

The PPO activity has the influence on the growth of plants, animals and microorganisms and it is known that compounds which inhibit the plant-derived PPO activity have generally the herbicidal activity. Thus, in order to effectively develop PPO inhibiting-type herbicides, there has been a need for a method for evaluating the ability of a compound to inhibit the PPO activity with simplicity.

## SUMMARY OF THE INVENTION

Under the aforementioned circumstances, the present inventors studied hard, which resulted in completion of the present invention.

The present invention provides:

1. A method for evaluating the ability of a compound to inhibit the protoporphyrinogen oxidase activity, which comprises the steps of:

(1) culturing a transformant expressing a protoporphyrinogen oxidase gene present in a DNA fragment in a medium containing substantially no protoheme compounds in each comparative system of the presence and absence of a test compound to measure a growth rate of the transformant under each condition, said transformant being resulted from a host cell deficient in the growing ability based on the protoporphyrinogen oxidase activity transformed with the DNA fragment in which a promoter functionable in the host cell and a protoporphyrinogen oxidase gene are operatively linked, and

(2) determining the ability of the compound to inhibit the protoporphyrinogen oxidase activity by comparing the growth rates (hereinafter referred to as "the present method").

2. A method for evaluating the ability of a compound to inhibit the protoporphyrinogen oxidase activity, which comprises the steps of:

(1) culturing a transformant expressing a protoporphyrinogen oxidase gene present in a DNA fragment in a medium containing substantially no protoheme compounds in each comparative system of the presence and absence of a test compound

to measure a growth rate of the transformant under each condition, said transformant being resulted from a host cell deficient in the growing ability based on the protoporphyrinogen oxidase activity transformed with the DNA fragment in which a promoter functionable in the host cell, a protoporphyrinogen oxidase gene and a terminator functionable in the host cell are operatively linked, and

(2) determining the ability of the compound to inhibit the protoporphyrinogen oxidase activity by comparing the growth rates.

3. A method for evaluating the ability of a compound to inhibit the protoporphyrinogen oxidase activity, which comprises the steps of:

(1) culturing a transformant expressing a protoporphyrinogen oxidase gene present in the following (a) DNA fragment in a medium containing substantially no protoheme compounds in each comparative system of the presence and absence of a test compound to measure a growth rate of the transformant under each condition, said transformant being resulted from a host cell deficient in the growing ability based on protoporphyrinogen oxidase activity transformed with

(a) the DNA fragment in which a promoter functionable in the host cell and controllable in its transcriptional activity, and a protoporphyrinogen oxidase gene are operatively linked, and

(b) a DNA fragment in which a gene being capable of controlling the transcriptional activity of the promoter in the above DNA fragment and a promoter having the transcriptional activity not controllable by the gene and functionable in the host cell are operatively linked, and

(2) determining the ability of the compound to inhibit the protoporphyrinogen

oxidase activity by comparing the growth rates.

4. A method for evaluating the ability of a compound to inhibit the protoporphyrinogen oxidase activity, which comprises the steps of:

(1) culturing a transformant expressing a protoporphyrinogen oxidase gene present in the following (a) DNA fragment in a medium containing substantially no protoheme compounds in each comparative system of the presence and absence of a test compound to measure a growth rate of the transformant under each condition, said transformant being resulted from a host cell deficient in the growing ability based on the protoporphyrinogen oxidase activity transformed with

(a) the DNA fragment in which a promoter functionable in the host cell and controllable in its transcriptional activity, a protoporphyrinogen oxidase gene and a terminator functionable in the host cell are operatively linked, and

(b) a DNA fragment in which a gene being capable of controlling the transcriptional activity of the promoter in the above DNA fragment, a promoter having the transcriptional activity not controllable by the gene and functionable in the host cell, and a terminator functionable in the host cell are operatively linked, and

(2) determining the ability of the compound to inhibit the protoporphyrinogen oxidase activity by comparing the growth rates.

5. The method according to 1 or 3, which is characterized in that the protoporphyrinogen oxidase gene is a protoporphyrinogen oxidase gene derived from an animal or a plant.

6. The method according to 1 or 3, which is characterized in that the protoporphyrinogen oxidase gene is a protoporphyrinogen oxidase gene derived from a

rat or *Chlamydomonas reinhardtii*.

7. The method according to 1 or 3, which is characterized in that the host cell is a microorganism.

8. A rat-derived gene encoding a protein having the protoporphyrinogen oxidase activity.

9. A protoporphyrinogen oxidase gene encoding a protein having the amino acid sequence shown by SEQ ID: No.1.

10. A gene encoding a protein having the protoporphyrinogen oxidase activity and having the amino acid sequence in which one or several amino acids are deleted, substituted, modified or added in the amino acid sequence shown by SEQ ID: No.1.

11. A protoporphyrinogen oxidase gene having the nucleotide sequence encoding the amino acid sequence shown by SEQ ID: No.1.

12. A protoporphyrinogen oxidase gene having the nucleotide sequence shown by SEQ ID: No.2.

13. A DNA fragment having a partial nucleotide sequence of the protoporphyrinogen oxidase gene of any one of 8 to 12.

14. A vector which comprises the protoporphyrinogen oxidase gene of any one of 8 to 12.

15. A transformant which is characterized in that the vector of 14 is introduced in to a host cell.

16. The transformant according to 15, wherein the host cell is a microorganism.

17. The transformant according to 15, wherein the host cell is a plant.



18. A *Chlamydomonas reinhardtii*-derived gene encoding a protein having the protoporphyrinogen oxidase activity.

19. A protoporphyrinogen oxidase gene encoding a protein having the amino acid sequence shown by SEQ ID: No.9.

20. A gene encoding a protein having the protoporphyrinogen oxidase activity and having the amino acid sequence in which one or several amino acids are deleted, substituted, modified or added in the amino acid sequence shown by SEQ ID: No.9.

21. A protoporphyrinogen oxidase gene having the nucleotide sequence encoding the amino acid sequence shown by SEQ ID: No.9.

22. A protoporphyrinogen oxidase gene having the nucleotide sequence shown by SEQ ID: No.10.

23. A DNA fragment having a partial nucleotide sequence of the protoporphyrinogen oxidase gene of any one of 18 to 22.

24. A vector which comprises the protoporphyrinogen oxidase gene of any one of 18 to 22.

25. A transformant which is characterized in that the vector of 24 is introduced in a host cell.

26. The transformant according to 25, wherein the host cell is a microorganism.

27. The transformant according to 25, wherein the host cell is a plant.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a method for constructing a vector containing a full length rat-derived protoporphyrinogen oxidase cDNA.

[PPO fragment 1] shows a DNA fragment obtained by a polymerase chain reaction of Example 2, [PPO fragment 2] shows a DNA fragment harbored by a clone obtained in Example 1, [PPO] shows a rat-derived protoporphyrinogen oxidase cDNA, and other symbols show a restriction enzyme recognition site.

Fig. 2 shows a method for constructing a protoporphyrinogen expression vector which is constructed for expressing a rat-derived protoporphyrinogen oxidase by introducing it into *Escherichia coli*.

[PPO] shows a rat-derived protoporphyrinogen oxidase cDNA, [*lacZ*] shows a beta-galactosidase gene, and other symbols show a restriction enzyme recognition site.

Fig. 3 shows a method for constructing a *lac* repressor expression vector which is constructed for expressing a *lac* repressor by introducing it into *Escherichia coli*.

Fig. 4 shows the structure of a compound which was subjected to a test on the ability of a compound to inhibit the rat-derived protoporphyrinogen oxidase activity.

Fig. 5 shows a method for constructing a protoporphyrinogen oxidase expression vector for direct introduction which is constructed for expressing a rat-derived protoporphyrinogen oxidase by introducing it into a plant cell.

[35S] shows the cauliflower mosaic virus-derived 35S promoter, [NOS] shows the *Agrobacterium*-derived nopaline synthase terminator, [PPO] shows a rat-derived protoporphyrinogen oxidase cDNA, and other symbols show a restriction enzyme recognition site.

Fig. 6 shows a method for constructing a protoporphyrinogen oxidase expression vector for indirect introduction which is constructed for a rat-derived protoporphyrinogen oxidase by introducing it into a plant cell.

[35S] shows the cauliflower mosaic virus-derived 35S promoter, [NOS] shows the *Agrobacterium*-derived nopaline synthase terminator, [PPO] shows a rat-derived protoporphyrinogen oxidase cDNA, [NTPII] shows a kanamycin-resistant gene, [NOSp] shows the *Agrobacterium*-derived nopaline synthase promoter, [LB] and [RB] show the *Agrobacterium* T-DNA-derived left border nucleotide sequence and right border nucleotide sequence, respectively, and other symbols show a restriction enzyme recognition site.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention will be described in detail.

First, the present method will be described.

A PPO gene used in the present method may be any ones encoding a protein having the PPO activity upon expression in the host cell and, for example, may be selected from PPO genes derived from plants and animals. More particularly, there are PPO genes derived from Dicotyledonous plants such as *Arabidopsis*, soybean, oil seed rape, sugar beat, potato and tobacco, Monocotyledonous plants such as corn, rice, wheat, barley, oat, rye, sugar cane and sorghum, algae such as *Chlamydomonas reinhardtii* and *Chlorella*, mammals such as mouse, rat and human, fishes sich as trout, bluegill, carp, cyprinodont, guppy, zebra fish and fathead minnow, insects such as fly, mosquito, cockroach, greasy grind, dragonfly and silkworm moth.

As used herein, "a promoter functionable in a host cell" used in the present method is a DNA fragment having the transcriptional activity in a host cell being transformed and includes, for example, *Escherichia coli* lactose operon promoter (*lacP*),

tryptophan operon promoter (*trpP*), arginine operon promoter (*argP*), galactose operon promoter (*galP*), *tac* promoter, T7 promoter, T3 promoter,  $\lambda$  phage promoter ( $\lambda$ -pL,  $\lambda$ -pR), yeast alcohol dehydrogenase gene (ADH) promoter, adenovirus major late (Ad.ML) promoter, SV40 early promoter, baculovirus promoter, nopaline synthase gene (NOS) promoter, octopine synthase gene (OCT) promoter, cauliflower mosaic virus (CaMV)-derived 19S and 35S promoters, phenylalanine ammonia lyase (PAL) gene promoter, chalcone synthase (CHS) gene promoter and the like.

In addition, "a terminator functionable in a host cell" used if necessary includes any DNA fragments having the transcription terminating activity in a host cell being transformed. Examples thereof include *Escherichia coli* lactose operon terminator, arginine operon terminator, galactose operon terminator, yeast HIS terminator, ADH1 terminator, SV40 early splicing region, nopaline synthase gene (NOS) terminator, garlic virus GV1 and GV2 terminators and the like.

As used herein, "a DNA fragment are operatively linked " means a DNA fragment in which these promoter, and, if necessary, these terminator, are linked so that the above PPO gene is expressed under the control of the above promoter, and, if necessary, the above terminator, in a host cell into which they are to be introduced (hereinafter referred to as "expression DNA fragment").

The above expression DNA fragment is introduced into a host cell deficient in the growing ability based on the PPO activity to obtain a transformant expressing the introduced PPO gene.

A host cell being transformed, that is, deficient in the growing ability based on

the PPO activity may be any host cells deficient in the PPO activity necessary for growth and, from a viewpoint of simple culturing, microorganisms are preferable. Examples of microorganisms deficient in the PPO activity necessary for growth include PPO gene (*hemG* locus)-deficient mutant *Escherichia coli* strain VSR 751 described in K.

Miyamoto, K. Nakahigashi, K. Nishimura, T. Nakayashiki and H. Inokuchi, (1991) Journal of Molecular Biology, vol.219, pp393-398 and K. Nishimura, T. Nakayashiki and H. Inokuchi, (1993) Gene, vol.133, pp109-113, PPO gene (*hemG* locus)-deficient mutant *Escherichia coli* strain BT3 described in F. Yamamoto, H. Inokuchi and H. Ozeki, (1988) Jpn. J. Genet., vol.63, pp237-249, PPO gene (*hem14-1* locus)-deficient mutant yeast strain *hem14-1* described in J.M.Gamadro, D.Urban-Grimal and P.Labbe (1982) Biochem. Biophys. Res. Commun., vol.106, pp724-730, and the like.

The above expression DNA fragment incorporated into a vector may be introduced into a host cell. The vector containing the expression DNA fragment may be introduced into a host cell according to the conventional genetic engineering method. For example, when the host cell is a microorganism, the vector may be introduced into a cell of the microorganism with a calcium chloride method, an electroporation method (Methods in Electroporation: Gene Pulser/*E. coli* Pulser System, Bio-Rad Laboratories, 1993) and the like. In addition, when the host cell is a plant, the vector may be introduced into a cell of the plant with an *Agrobacterium* infection method (JP-B-2-58917 (examined) and JP-A-60-70080 (laid-opened)), an electroporation into a protoplast method (JP-A-60-251887 (laid-opened) and JP-A-5-68575 (laid-opened)), a particle gun method (JP-A-5-508316 (laid-opened) and JP-A-63-258525 (laid-opened)) and the like. A transformant expressing the introduced PPO gene may be obtained by

culturing the host cell with the introduced vector in a medium containing a growth inhibitor corresponding to a selectable marker locus-linked on the vector and a medium containing substantially no protoheme compounds, and isolating a clone which can grow in the medium.

The transformant thus obtained is first cultured in a medium containing substantially no protoheme compounds in each comparative system of the presence at a variety of concentrations and absence of a test compound to measure a growth rate of the transformant under each condition. Next, the PPO activity inhibiting ability of the test compound to inhibit the PPO activity is determined by comparing the growth rates based on the each comparative system of contact and non-contact of the transformant and the test compound. The comparison of the growth rates is performed, for example, by obtaining the growth inhibiting rate by calculating a ratio of the growth rate in a system of the presence of the test compound relative to the growth rate in a system of the absence of the test compound, and comparing the growth inhibiting rates regarding a plurality of test compounds and, based on the results thereof, the higher or lower ability for inhibiting PPO activity may be determined. More particularly, for example, a concentration of a test compound at which the growth is inhibited by 50% is obtained from the growth rate of the transformant in the system of the addition of a compound (that is, of the presence of the test compound) under the conditions that the growth rate of the transformant is 100% in the system of no addition of a compound (that is, of the absence of the test compound). The test compound having the lower concentration may be determined to be higher in the ability for inhibiting PPO activity than the test compound having the higher concentration. Alternatively, a compound known to

inhibit the PPO activity (positive control) is simultaneously subjected to an experiment as one of test compounds, which may serve as an index for the growth inhibiting rate of each test compound (for example, a compound concentration at which the growth is inhibited by 50%).

As used herein, "a medium containing substantially no protoheme compounds" means a medium not containing any protpheme compounds or a medium which may contain protoheme compounds at such an amount that a host cell deficient in the growing ability based on the PPO activity can not recover the growing ability to the same level as that of the corresponding non-deficient host cell (that is, a host cell not deficient in the growing ability based on the PPO activity) regardless of the presence of protoheme compounds in the medium. The examples thereof include an artificial medium not containing any protoheme compounds such as protoporphyrin, protohemin and the like at all, a medium containing natural extracts (for example, yeast extract, Malt extract and the like), and the like. From a viewpoint of the precision of the present method, it is preferable that an amount of the protoheme compounds present in a medium is zero or as low as possible. In addition, protoheme compounds include protoheme and its derivatives and these compounds mean a compound having the action of recovering the growing ability of a host cell deficient in the growing ability based on the PPO activity.

In order to confirm that the growth inhibition by a test compound is due to the inhibition of PPO activity, it can be easily determined by performing the culturing of the above transformant in a medium containing substantially protoheme compounds such as protoporphyrin, protohemin and the like, and confirming the growth of the transformant.

In addition, if needed, the growth rate of the transformant may be conveniently adjusted by the relationship between an expressed amount of PPO gene and a concentration of a test compound.

In the present method, the expressed amount of PPO gene in the transformant may vary by using "a promoter functionable in a host cell and controllable in its transcriptional activity" as a promoter for the aforementioned expression DNA fragment, and introducing into the host cell "a DNA fragment in which a gene capable of controlling the transcriptional activity of a promoter in the expression DNA fragment and a promoter having the transcriptional activity not-controllable by the gene and functionable in the host cell, and an optionally used terminator functionable in the host cell are operatively linked" (hereinafter referred to as "regulatory DNA fragment"). For example, when evaluation of a test compound having the lower water-solubility and that of a small amount of a test compound are performed, by adjusting an expressed amount of PPO gene in the transformant to be small, evaluation may be performed with the higher precision.

On the other hand, when evaluation of a test compound having the higher ability for inhibiting PPO activity are performed, by adjusting an expressed amount of PPO gene in the transformant to be increased, evaluation may be performed with the higher precision.

As used herein, "a gene capable of controlling the transcriptional activity of a promoter in the expression DNA fragment" (hereinafter referred to as "a regulatory gene") means a gene having the function of inhibiting or promoting the transcriptional activity of "a promoter functionable in the host cell and controllable in its transcriptional



activity" as a promoter for the expression DNA fragment. When an expressed amount of PPO gene is to be decreased, a gene which inhibits the transcriptional activity of a promoter in the expression DNA fragment may be used, while when an expressed amount of PPO gene is to be increased, a gene which promotes the transcriptional activity of the promoter may be used. More particularly, examples of a combination for inhibiting the transcriptional activity include a combination of *Escherichia coli* lactose operon promoter and *lacI*, that of arginine operon promoter and *argR*, that of galactose operon promoter and *GalR* and the like. On the other hand, examples of a combination for promoting the transcriptional activity include a combination of *Escherichia coli* lactose operon promoter and *crp*, that of *Klebsiella pneumoniae* maltose regulon and *malT* and the like.

"A promoter having the transcriptional activity not-controllable by the regulatory gene and functionable in a host cell", that is, a promoter for the regulatory DNA fragment may be any promoters having no influence by a gene which they themselves control. For example, in a case of a promoter to be combined with a repressor gene for the above lactose operon, its own promoter, nopaline synthase enzyme gene (NOS) promoter and the like may be used.

The above expression DNA fragment and the regulatory DNA fragment may be introduced into a host cell by incorporating into the same or different vector and these DNA fragments may be incorporated into chromosome(s) of the host cell.

The present method may be utilized for screening medications having the ability for inhibiting the malignant cell growth in addition to evaluating the potency of a phototrophic herbicide, screening a compound having the herbicidal activity.

When the present method is utilized for evaluating the potency of a phototrophic herbicide or screening a compound having the herbicidal activity, the present method is performed, for example, using PPO genes derived from higher plants such as *Arabidopsis*, PPO genes derived from algae such as *Chlamydomonas reinhardtii*. As a result, a compound inhibiting the growth of the transformant into which a PPO gene derived from higher plants or algae is introduced is potentially useful as an active ingredient of a herbicide.

Next, a gene which may be used for the present method will be described in detail.

Examples of a rat-derived gene encoding a protein having the PPO activity (hereinafter referred to as "the present rat PPO gene") are (1) a rat-derived gene having about 1.64 kbp of length, for example, a PPO gene encoding a protein having the amino acid sequence shown by SEQ ID: No.1, (2) a gene encoding a protein having the PPO activity and having the amino acid sequence in which one or several amino acids are deleted, substituted, modified or added in the amino acid sequence shown in SEQ ID: No.1. More particularly, for example, mention be made of a PPO gene having the nucleotide sequence shown by SEQ ID: No.2.

Examples of a *Chlamydomonas reinhardtii*-derived gene encoding a protein having the PPO activity (hereinafter referred to as "the present *Chlamydomonas reinhardtii* PPO gene") are (1) a *Chlamydomonas reinhardtii*-derived gene having about 2 kbp of length, for example, a PPO gene encoding a protein having the amino acid sequence shown by SEQ ID: No.9, (2) a gene encoding a protein having the PPO activity and having the amino acid sequence in which one or several amino acids are

deleted, substituted, modified or added in the amino acid sequence shown in SEQ ID:

No.9. More particularly, for example, mention be made of a PPO gene having the nucleotide sequence shown by SEQ ID: No.10.

The present rat PPO gene is obtained, for example, according to the following method.

RNA is extracted from rat tissues such as liver, kidney and the like according to a method described in Labomanual Genetic Engineering, Suppl. Edition, edited by Matsumura, published by Maruzen K.K., pp76-77, 1990. The extracting procedures may be carried out utilizing a commercially available RNA extracting kit, for example, ISOGEN (Nippon Gene).

The resultant RNA is subjected to the manipulation according to the attached manual using a commercially available poly(A) RNA fractionating kit such as BIOMAG mRNA Purification kit (Perceptive Biosystems) to prepare poly(A) RNA. The resulting poly(A) RNA is subjected to the manipulation according to a method described in Cloning and Sequence: Plant Biotechnology Experimental Manual, edited by Watanabe and Sugiura, published by Nosonbunka Company, pp74-103 (1989) to make cDNA library. This cDNA library making manipulation may utilize a commercially available cDNA library making kit such as ZAP-cDNA Gigapack Cloning Kit (STRATAGENE).

From thus made cDNA library or a commercially available cDNA library such as a rat liver-derived cDNA liver manufactured by STRATAGENE, DNA may be prepared by a method described in Molecular Cloning 2nd edition (authors: J. Sambrook, E.F.Frisch, and T.Maniatis; Cold Spring Harbor Laboratory Press, 1989) 2.60 - 2.81.

By using thus prepared DNA as a template, a polymerase chain reaction may be performed using an oligonucleotide having the nucleotide sequence shown by SEQ ID: No.3 and that having the nucleotide sequence shown in SEQ ID: No.4 as a primer to amplify a DNA fragment having a partial nucleotide sequence of PPO gene. This amplified DNA fragment having a partial nucleotide sequence of a PPO gene may be cloned into a plasmid using the conventional method described in Labomanual Genetic Engineering, Suppl. Edition, edited by Matsumura, published by Maruzen K.K., pp117-120 (1990) and a commercially available DNA cloning kit such as TA cloning kit (manufactured by Invitrogen) to serve for determining nucleotide sequence.

Determination of the nucleotide sequence may be performed by a dideoxy method described, for example, in Molecular Cloning 2nd edition (authors: J.Sambrook, E.F.Frisch and T.Maniatis; Cold Spring Harbor Laboratory Press), 13.42-13.74 (1989).

Determination of the nucleotide sequence according to dideoxy method may be performed utilizing a sequencing kit using fluorescently-labeled dideoxynucleotide (more particularly, for example, Dye terminator cycle sequencing kit (manufactured by PE Applied Biosystems) and using a commercially available autosequencer such as DNA Sequencer 373S (manufactured by PE Applied Biosystems).

Thus, the DNA fragment having a partial nucleotide sequence of a rat PPO gene (hereinafter referred to as "the present rat PPO DNA fragment") can be obtained.

One example of the present rat PPO DNA fragment is a gene fragment having the nucleotide sequence shown in nucleotide Nos. 639 to 1333 shown by SEQ ID: No.2.

The present rat PPO DNA fragment may be utilized in making a PPO gene-specific primer used in a polymerase chain reaction described below or used as a probe for

detecting a PPO gene in a hybridization method described below.

Based on the present rat PPO DNA fragment, the present rat PPO gene having entire nucleotide sequence encoding PPO may be obtained according to (1) polymerase chain reaction method or (2) hybridization method.

In order to obtain a PPO gene by utilizing a polymerase chain reaction, a primer having about 15bp to about 40bp nucleotide sequence among the nucleotide sequence of the present rat PPO DNA fragment (primer for amplifying 3' downstream region) and about 15bp to about 40bp nucleotide sequence among the nucleotide sequence complementary to the base sequence of the present rat PPO DNA fragment (primer for amplifying 5' upstream region) are first prepared. Examples of the primer for amplifying 3' downstream region are a gene fragment having the nucleotide sequence shown in basenucleotide Nos.1175 to 1198 by SEQ ID: No.2 and the like, and examples of the primer for amplifying 5' upstream region are a gene fragment having the nucleotide sequence complementary to the nucleotide sequence shown in nucleotide Nos.776 to 799 by SEQ ID: No.2 and the like.

Then, a polymerase chain reaction is performed using as a template a DNA fragment having an adapter DNA added at an end of a rat-derived DNA or a DNA of a vector having rat-derived DNA fragment inserted therein and using a combination of a primer having a partial nucleotide sequence of an adapter DNA or a primer having a partial nucleotide sequence of a vector DNA and a primer for amplifying 5' upstream region and a combination of a primer having a partial nucleotide sequence of an adapter DNA or a primer having a partial nucleotide sequence of a vector DNA and a primer for amplifying 3' downstream region to amplify a DNA fragment containing 5' upstream

nucleotide sequence of the present rat PPO DNA fragment and a DNA fragment having 3' downstream nucleotide sequence of the present rat PPO DNA fragment. The DNA fragment having an adapter DNA added at an end of a rat-derived DNA which is used as a template may be prepared by adding as an adapter DNA a polymer of a nucleotide such as cytosine to a rat-derived cDNA prepared above with terminal deoxynucleotidyl transferase or adding a commercially available adapter of a kit for RACE (rapid amplification of cDNA ends) reaction such as an adapter attached to Marathon cDNA amplification kit manufactured by Clontech to a rat-derived cDNA. In addition, one example of the primer having a partial nucleotide sequence of an adapter DNA is a primer specific for an adapter attached to a commercially available RACE (rapid amplification of cDNA ends) reaction kit (for example, AP-1 primer and AP-2 primer attached to Marathon cDNA amplification kit manufactured by Clontech). As the primer having a partial sequence of a vector DNA, when a vector is derived from  $\lambda$  phage, primers specific for an arm region of  $\lambda$  phage such as  $\lambda$  gtII reverse primer and  $\lambda$  gtII forward primer may be used.

Two kinds of thus amplified DNA fragments may be cloned into a plasmid by using the conventional method described in Labomanual Genetic Engineering, Suppl. Edition, edited by Matsumura, published by Maruzen K.K., pp117-120 (1990) or a commercially available DNA cloning kit such as TA cloning kit (manufactured by Invitrogen) to serve for determination of the nucleotide sequence. Determination of the nucleotide sequence may be performed by a dideoxy method described in Molecular Cloning 2nd edition (authors: J.Sambrook, E.F.Frisch and T.Maniatis; Cold Spring

Harbor Laboratory Press), 13.42-13.74 (1989). Determination of the nucleotide sequence by a dideoxy method may be performed by utilizing a sequencing kit using a fluorescently labeled dideoxynucleotide such as Dye terminator cycle sequencing kit (manufactured by PE Applied Biosystems) and using a commercially available autosequencer such as DNA Sequencer 373S (manufactured by PE Applied Biosystems). The regions between the thus determined nucleotide sequence and the nucleotide sequence of the present rat PPO DNA fragment which was referenced upon manufacturing the above primer are overlaid to ligate these nucleotide sequences to make one base sequence. Whether or not an entire region of open reading frame encoding PPO is contained in the thus made nucleotide sequence may be studied by investigating open reading frame using a commercially available gene analyzing software such as GENETYX (manufactured by SDC). When a 1.6kbp or more entire open reading frame is not contained in the determined nucleotide sequence and translation initiation codon or translation termination codon are not contained therein, it is determined that the whole nucleotide sequence of the full length PPO gene is not contained in the nucleotide sequence, and steps for obtaining a gene fragment by a polymerase chain reaction is repeated until PPO gene having the whole nucleotide sequence encoding PPO is obtained. In addition, when open reading frame which is found in the nucleotide sequence determined as described above is missing in either in 5' upstream or 3' downstream side of the determined nucleotide sequence, that is, when a translation initiation codon or translation termination codon is not contained in the open reading frame, steps for obtaining a gene fragment by the polymerase chain reaction same as above may be repeated for obtaining a DNA fragment containing the nucleotide

sequence on the missing's side.

Based on the information of the nucleotide sequence thus determined, a primer containing the nucleotide sequence near translation initiation codon in the nucleotide sequence of a PPO gene (N-terminal primer) and a primer containing the nucleotide sequence complementary to the nucleotide sequence near translation termination codon (C-terminal primer) are made, and a polymerase chain reaction may be performed using a rat-derived DNA as a template and using the above both terminal primers to amplify the present rat PPO gene having the entire nucleotide sequence encoding PPO, which may be cloned. Examples of the N-terminal primer include a primer having the nucleotide sequence shown by SEQ ID: No. 5 and a primer having the nucleotide sequence shown by SEQ ID: No. 7, and examples of the C-terminal primer include a primer having the nucleotide sequence shown by SEQ ID: No. 6 and a primer having the nucleotide sequence shown by SEQ ID: No. 8. Alternatively, the DNA fragments obtained by the above steps for obtaining the gene fragment may be ligated with an restriction enzyme recognition site present in the overlapping regions to obtain the present rat PPO gene having the entire nucleotide sequence encoding PPO.

(2) In order to obtain the present rat PPO gene by utilizing a hybridization method, a DNA fragment may be identified by hybridizing a probe of the present rat PPO DNA fragment to a rat-derived DNA and the identified DNA fragment may be isolated.

A probe used for a hybridization method may be prepared by labeling the present rat PPO DNA fragment obtained as described above with a labeling compound by the conventional method described in Molecular Cloning 2nd edition (authors:



J.Sambrook, E.F.Frisch and T. Maniatis; Cold Spring Harbor Laboratory Press), 10.6-10.26 (1989). A labeling compound such as a compound containing a radioactive element (hereinafter referred to as "RI") and a fluorescent reagent and the like may be used. Labeling with a compound containing RI may be performed using [ $\alpha$ - $^{32}$ P] dCTP (manufactured by Amersham) and a random prime labeling kit (manufactured by Boehringer Mannheim) according to the protocol attached to the kit. In addition, non-RI labeling may be performed using DIG-High Prime DNA Labeling and Detection Starter Kit I (Boehringer Mannheim) and attached reagents according to the attached protocol.

As a rat-derived DNA for hybridizing with a probe, a rat cDNA library and the like may be used which is obtained by ligating a cDNA derived from a rat to a vector with a suitable adaptor, derived from  $\lambda$  phage and the like and packaging the cDNA into phage particles. Such the gene library may be prepared according to a method, for example, described in Cloning and Sequencing: Plant Biotechnology Experimental Manual, edited by Watanabe and Sugiura, published by Nosonbunka, pp74-103 (1989). Alternatively, the rat gene libraries sold by Clontech, STRATAGENE and the like may be used.

In order to isolate the present gene, for example, colony hybridization, plaque hybridization and the like to the aforementioned rat gene library may be performed using the probe prepared by the aforementioned method. These may be performed according to a method described in Recombinant DNA Experiment (edited by J.R.Dillon, A.Nasim and E.R.Nestmann, published by Tokyo kagakudozin), pp98-101 (1987) or the conventional method described in Molecular Cloning 2nd edition (authors:

J.Sambrook, E.F.Frisch and T.Maniatis; Cold Spring Harbor Laboratory Press), pp1.90-1.104 or 2.108-2.117 (1989). Determination of the nucleotide sequence of the DNA fragment incorporated in the resultant clone may be performed by a dideoxy method described in Molecular Cloning 2nd edition (authors: J.Sambrook, E.F.Frisch and T.Maniatis, Cold Spring Harbor Laboratory Press), pp13.42-13.74 (1989). The determination of the nucleotide sequence by a dideoxy method may be performed by utilizing a sequencing kit using fluorescently labeled dideoxynucleotide such as Dye terminator cycle sequencing kit (manufactured by PE Applied Biosystems) and using a commercially available autosequencer such as DNA Sequencer 373S (manufactured by PE Applied Biosystems). The determined nucleotide sequence may be analyzed using a commercially available gene analyzing software such as GENETYX (SDC) to reveal the entire nucleotide sequence of the present rat PPO gene.

Next, the present *Chlamydomonas reinhardtii*-derived PPO gene is obtained by the following method. The fundamental manipulation may be performed according to the aforementioned description for obtaining the present rat PPO gene.

*Chlamydomonas reinhardtii* is cultured to collect cells and RNA is extracted from the collected cells. A cDNA library is made from the resultant RNA according to a method for obtaining the present rat PPO gene to prepare its DNA.

By using the DNA thus prepared as a template, a polymerase chain reaction may be performed using as a primer an oligonucleotide having the nucleotide sequence shown by SEQ ID: No.11 and that having the nucleotide sequence shown by SEQ ID: No.12 to amplify a DNA fragment having a *Chlamydomonas reinhardtii* PPO gene. The amplified DNA fragment having the nucleotide sequence of PPO gene may be

cloned into a plasmid to serve for determination of the nucleotide sequence.

Thus obtained cDNA of the present rat PPO gene or the present rat PPO DNA fragment, or the cDNA of the present *Chlamydomonas reinhardtii* PPO gene may be used to obtain a genome DNA clone of the present rat PPO gene or the present *Chlamydomonas reinhardtii* PPO gene, the nucleotide sequence of which may be determined. First, in the case of the genome DNA clone of the present rat PPO gene, for example, rat tissues such as liver, kidney and the like are taken and a genome DNA is extracted from the taken tissues by manipulations according to a method described in Labomanual Genetic Engineering, Suppl. Edition, edited by Matsumura, published by Maruzen K.K., pp76-77 (1990). On the other hand, in the case of a genome clone of the present *Chlamydomonas reinhardtii* PPO gene, for example, *Chlamydomonas reinhardtii* is cultured to collect cells and a genome DNA is extracted from the collected cells according to the similar procedures. The extracting procedures may be performed using a commercially available DNA extracting kit such as ISOTISSUE (Nippon Gene) according to the protocol attached to the kit to obtain a genome DNA. After the genome DNA is cut with a suitable restriction enzyme, the resultant genome DNA fragment is fractionated according to a NaCl density gradient centrifugation method described in Cloning and Sequencing: Plant Biotechnology Experimental Manual, edited by Watanabe and Sugiura, published by Nosonbunka, pp276-279 (1989). In general, conveniently, a fraction containing a genome DNA fragment having a size suitable for incorporating into a vector, particularly, a fraction containing 9kbp to 23kbp genome DNA fragment when a phage vector is used, a fraction containing 30kbp to 42kbp genome DNA fragment when a cosmid vector is used is selected from the

fractionated respective genome DNA fragments. A genome library is made using the resultant genome DNA fragments and performing the procedures according to a method described in Cloning and Sequencing: Plant Biotechnology Experimental Manual, edited by Watanabe and Sugiura, published by Nosonbunka, pp96-103 and 280 -284 (1989) or using a commercially available DNA library making kit such as Lambda EMBL3/Gigapack cloning kit (STRATAGENE) according to the attached protocol. The genome DNA library thus made, or a commercially available genome library such as rat genome library manufactured by STRATAGENE is screened by hybridization using as a probe a cDNA of the present rat PPO gene or the present rat PPO DNA fragment, or a cDNA of the present *Chlamydomonas reinhardtii* PPO gene according to a method described in Cloning and Sequence: Plant Biotechnology Experimental Manual, edited by Watanabe and Sugiura, published by Nosonbunka, pp106-147 (1989) to obtain a genome DNA clone having the nucleotide sequence of the present rat PPO gene, or the present *Chlamydomonas reinhardtii* PPO gene. The resultant genome DNA clone may be subcloned into a suitable vector for analyzing the nucleotide sequence such as a plasmid and the like according to a method described in Cloning and Sequence: Plant Biotechnology Experimental Mannual, edited by Watanabe and Sugiura, published by Nosonbunka, pp152-174 (1989), and sequenced by a dideoxy method and the like according to a primer extension method described in Molecular Cloning 2nd edition (authors; J. Sambrook, E.F.Frisch and T.Maniatis; Cold Spring Harbor Laboratory), pp13.15 (1989). Determination of the nucleotide sequence by dideoxy method may be performed using a commercially available kit such as Dye terminator cycle sequencing kit manufactured by PE Applied Biosystems and using a DNA

sequencer such as Model 373S of PE Applied Biosystems.

Further, by a primer extension method described in Bina-Stem Met et al., (1979) Proc. Natl. Acad. Sci. U.S.A., vol. 76, pp 731 and Sollner-Webb and R.H. Reeder, (1979) Cell, vol. 18, pp 485 or a S1 mapping method described in A.J. Berk and P.A. Sharp, (1978) Proc. Natl. Acad. Sci. U.S.A., vol. 75, pp 1274 and the like, each transcription initial point of the genome DNA of the present rat PPO gene or the present *Chlamydomonas reinhardtii* PPO gene may be decided. At about 1kb to about 10kb upstream from this transcription initiation point, there is a promoter sequence responsible for controlling the gene expression.

In addition, the present rat PPO gene and the present *Chlamydomonas reinhardtii* PPO gene can be utilized as a means for obtaining or generating a gene resistance to a PPO activity inhibiting agent known as a phototrophic herbicide. For example, it becomes possible to generate a new herbicide-resistant gene by introducing into a PPO gene a mutation which gives a change to the amino acid sequence of PPO encoded by the present rat PPO gene or the present *Chlamydomonas reinhardtii* PPO gene and screening a gene encoding the amino acid sequence of PPO showing phototrophic herbicide-resistance. More particularly, it becomes possible to generate a new phototrophic herbicide-resistant gene by inducing a random mutation in the nucleotide sequence of the present rat PPO gene or the present *Chlamydomonas reinhardtii* PPO gene to introduce a mutation into the amino acid sequence according to a method described in A. Greener, M. Callahan, Strategies, 1994, Vol. 7, pp 32 to 34, and the like. In addition, it also becomes possible to generate a new phototrophic herbicide-resistant gene by introducing a site-specific mutation into the nucleotide

sequence of the present rat PPO gene or the present *Chlamydomonas reinhardtii* PPO gene to alter the amino acid sequence according to a gapped duplex method described in W.Kramer, et al., Nucleic Acids Research, 1984, vol.12, pp9441 or W.Kramer, H.J.Frits, Methods in Enzymology, 1987, vol.154, pp350, or a Kunkel method described in T.A.Kunkel, Proc. of Natl. Acad. Sci. U.S.A., 1985, vol.82, pp488 or T.A.Kunkel, et. al., Methods in Enzymology, 1987, vol. 154, pp367, and the like. Further, it also becomes possible to generate a new phototrophic herbicide-resistant gene by making a gene encoding a chimera protein in which one or several partial amino acid sequences among the amino acid sequence of PPO encoded by the present rat PPO gene or the present *Chlamydomonas reinhardtii* PPO gene are substituted with a part of the amino acid sequence of the present *Chlamydomonas reinhardtii* PPO gene, the present rat PPO gene or PPO gene derived from other organisms. The effects of the thus made phototrophic herbicide-resistant gene (expresasion of herbicide resistance) can be effectively confirmed by, for example, introducing a generated herbicide-resistant gene into a microorganism defective in PPO or a microorganism having PPO sensitive to a subject herbicide, selecting a transformed microorganism, and treating the transformed microorganism with the subject herbicide to reselect a herbicide-resistant colony.

As a vector which comprises the present rat PPO gene or the present *Chlamydomonas reinhardtii* PPO gene, mention may be made of a plasmid made by cloning into pCR2.1 (Invitrogen) a PPO gene having the nucleotide sequence encoding the amino acid sequence shown by SEQ ID: No.1 or No.9 and the plasmid has the such the characteristics that a vector part is small and a copy number is large in *Escherichia coli* and, thus, is suitable for preparing DNA or analyzing the DNA structure.

A vector which can cause the present rat PPO gene or the present *Chlamydomonas reinhardtii* PPO gene to be expressed in a host cell can be constructed by, for example, inserting into a vector a DNA fragment in which (1) a promoter functionable in a host cell, and (2) the present rat PPO gene or the present *Chlamydomonas reinhardtii* PPO gene and, if necessary, (3) a terminator functionable in a host cell are operatively linked in an operative manner in the above order. As used herein, "operatively" means that the present rat PPO gene or the present *Chlamydomonas reinhardtii* PPO gene are ligated to a promoter (and, if necessary, a terminator) so that they are expressed under the control of the promoter (and the terminator) upon introduction of the vector into the host cell to transform the host cell.

As a "a promoter functionable in a host cell" is not limited to specified ones but may be any promoters having the transcriptional activity in a host cell to be transformed. For example, mention may be made of *Escherichia coli* lactose operon promoter, yeast alcohol dehydrogenase gene (ADH) promoter, adenovirus major late (Ad.ML) promoter, SV40 early promoter, baculovirus promoter, nopaline synthase gene (NOS) promoter, octopine synthase gene (OCT) promoter, cauliflower mosaic virus (CaMV)-derived 19S and 35S promoters, phenylalanine ammonia lyase (PAL) gene promoter, chalcone synthase (CHS) gene promoter and the like.

"A terminator functionable in a host cell" is not limited to specified ones but may be any terminators having the transcription terminating activity in a host cell to be transformed. For example, mention may be made of *Escherichia coli* lactose operon terminator, yeast HIS terminator, ADH1 terminator, SV40 early splicing region, nopaline synthase gene (NOS) terminator, garlic virus GV1, GV2 terminators and the

like.

A host cell can be transformed by introducing the above vector into the host cell. For example, when a host cell is a microorganism, the above vector can be introduced into a cell of the microorganism by the known means such as a calcium chloride method, an electroporation method (Methods in Electroporation: Gene Pulser/E. coli Pulser System, Bio-Rad Laboratories, 1993) and the like. On the other hand, when a host cell is a plant, a transformed plant cell can be obtained by introducing the above present vector into a cell of the plant by the known means such as an *Agrobacterium* infection method (JP-B-2-58917 (examined) and JP-A-60-70080 (laid-opened)), an electroporation to a protoplast method (JP-A-60-251887 (laid-opened) and JP-A-5-68575 (laid-opened)), or a particle gun method (JP-A-5-508316 (laid-opened) and JP-A-63-258525 (laid-opened)) to select a cell into which the present rat PPO gene or the present *Chlamydomonas reinhardtii* PPO gene is introduced. From the resultant transformed plant cell, a transformed plant body can be obtained by regenerating a transformed plant by a plant cell culturing method described in, for example, Plant Genetic Manipulation Manual: A method for generating A Transgenic Plant (author: Uchimiya, Kodansha Scientific, 1990, pp.27 to 55).

Furthermore, PPO activity of a host cell or sensitivity of a host cell to the inhibitor for PPO activity may be altered by transforming a host cell by introducing into the host cell a vector containing a DNA fragment in which (1) a promoter functionable in a host cell and (2) the present rat PPO gene or the present *Chlamydomonas reinhardtii* PPO gene and, if necessary, (3) a terminator functionable in a host cell using the present rat PPO gene or the present *Chlamydomonas reinhardtii* PPO gene.



As used herein, "a promoter functionable in a host cell" is not limited to specific ones but may be any promoters having the transcriptional activity in a host cell to be transformed. For example, mention may be made of *Escherichia coli* lactose operon promoter, yeast alcohol dehydrogenase gene (ADH) promoter, adenovirus major late (Ad.ML) promoter, SV40 early promoter, baculovirus promoter, nopaline synthase gene (NOS) promoter, octopine synthase gene (OCT) promoter, cauliflower mosaic virus (CaMV)-derived 19S and 35S promoters, phenylalanine ammonia lyase (PAL) gene promoter, chalcone synthase (CHS) gene promoter and the like.

"A terminator functionable in a host cell" is not limited to specified ones but may be any terminators having the transcription terminating activity in a host cell to be transformed. For example, mention may be made of *Escherichia coli* lactose operon terminator, yeast HIS terminator, ADH1 terminator, SV40 early splicing region, nopaline synthase gene (NOS) terminator, garlic virus GV1, GV2 terminators and the like.

By the above method for altering a host cell, for example, a resistance to a phototrophic herbicide targeting PPO can be imparted to a plant.

Other PPO genes can be obtained and utilized by the aforementioned method for obtaining the present rat PPO gene. For example, in order to obtain a rabbit-derived PPO gene, a commercially available rabbit cDNA library, primers, etc shown by SEQ ID: No.3 and No. 4 and the like may be used.

#### Examples

The following Examples illustrate the present invention in detail but do not limit the present invention.

#### Example 1 (Cloning of a rat PPO DNA fragment)

An oligonucleotide having the nucleotide sequence shown by SEQ ID: No.3 and that having the nucleotide sequence shown by SEQ ID: No.4 were prepared. The oligonucleotides were synthesized using a DNA synthesizer (PE Applied Systems: Model 394 DNA/RNA Synthesizer), and using as a solvent for synthesis a solvent for Model 394 DNA/RNA Synthesizer (PE Applied Systems) and using as a DNA synthesis reagent phosphoramidite reagents (PE Applied Systems) corresponding to adenine, cytosine, guanine and thymine. The synthesized oligonucleotides were purified by an oligonucleotide purifying cartridge (PE Applied Systems: OPC cartridge) and dried under reduced pressure to prepare oligonucleotides.

A library comprising  $\lambda$  ZAPII vector having inserted cDNA derived from a rat liver (manufactured by STRATAGENE) (hereinafter referred to as "rat cDNA library") was spread over several plates in NZCYM agar medium to amplify according to a method described in Molecular Cloning 2nd edition (authors: J.Sambrook, E.F.Frisch, T. Maniatis; Cold Spring Harbor Laboratory Press, 1989), 2.60-2.65 and phage particles were eluted with an SM buffer from the agar medium per each plate (hereinafter referred to as "amplified library"). A DNA was extracted from the amplified library using a DNA extracting kit (Lambda-TRAP PLUS: manufactured by Clontech) to prepare a phage cloned DNA. A polymerase chain reaction was performed using this phage cloned DNA as a template to amplify the DNA fragment. A reaction solution for polymerase chain reaction was prepared by taking 10 pmol of an oligonucleotide having

the nucleotide sequence shown by SEQ ID: No.3, 10 pmol of an oligonucleotide having the nucleotide sequence shown by SEQ ID: No. 4, 5  $\mu$ l 10 $\times$ PCR buffer (manufactured by TAKARA SHUZO CO., LTD), 0.25  $\mu$ l TaKaRa Taq (manufactured by TAKARA SHUZO CO., LTD), each 10 nmol of four kinds of nucleotides (dATP, dCTP, dGTP, dTTP: manufactured by TAKARA SHUZO CO., LTD), and 10ng of the phage clone DNA in a 0.5ml volume of a polymerase chain reaction tube and adding sterile distilled water to total 50  $\mu$ l. Each step of polymerase chain reaction was carried out under the following conditions: The first cycle comprising a denaturing step holding a temperature at 95  $^{\circ}$ C for 1 minute, an annealing step holding a temperature at 55 $^{\circ}$ C for 2 minutes, and an extension step with a DNA polymerase holding a temperature at 72 $^{\circ}$ C for 3 minutes was performed once, and the second cycle comprising a denaturing step holding a temperature at 95 $^{\circ}$ C for 1 minute, an annealing step holding a temperature at 55 $^{\circ}$ C for 1.5 minutes, and an extension step with a polymerase holding a temperature at 72 $^{\circ}$ C for 2 minutes was performed 34 times. After completion of the polymerase chain reaction, the reaction solution was analyzed on an agarose gel electrophoresis to select an amplified library from which about 700bp amplified DNA fragment is detected.

Next, *Escherichia coli* was infected with the selected amplified library and pBluescript vector containing a rat cDNA was prepared according to the description of manual attached to the above rat cDNA library, which was transformed into *Escherichia coli* to make an *Escherichia coli* library. Then, this *Escherichia coli* library was used to make a membrane for hybridization according to a method described in Recombinant

DNA Experiment (edited by J.R.Dillon, A Nasim, E.R.Nestmann, Tokyo Kagakudozin, 1987), pp98 to 100. Further, this membrane was used to perform hybridization using DIG-High Prime DNA Labeling and Detection Starter Kit I (manufactured by Boehringer Mannheim) and reagents attached thereto and according to the protocol attached thereto. As a probe, a DNA fragment having the nucleotide sequence shown by nucleotide Nos. 639 to 1333 in SEQ ID; No. 2. As a result, two clones which strongly hybridize with the probes were obtained.

The nucleotide sequence of the DNA fragment harbored by the resultant clone was determined using Dye terminator cycle sequencing kit (manufactured by PE Applied Systems) and a DNA sequencer 373S (manufactured by PE Applied Systems). As a result, the nucleotide sequence shown by nucleotide Nos.270 to 1636 in SEQ ID: No. 2 was revealed and it was found that the resultant two clones harbor the DNA fragments having the same nucleotide sequence. In addition, since a translation initiation codon is missing in the determined base sequence, a PPO gene encoded in the cloned DNA fragment was found to lack a 5' upstream region containing a translation initiation codon.

#### Example 2 (Cloning of a full length PPO gene)

In order to clone a 5' upstream region of a PPO gene which is missing in the PPO DNA fragment obtained in Example 1, a polymerase chain reaction was performed using as a template a DNA extracted from a rat cDNA library to amplify the DNA fragment. A reaction solution for polymerase chain reaction was prepared by taking 10 pmol of an oligobase having the nucleotide sequence shown by SEQ ID: No.4, 10pmol T3 primer (manufactured by TAKARA SHUZO CO., LTD), 0.5  $\mu$ l TaKaRa LATaq

(manufactured by TAKARA SHUZO CO., LTD), 5  $\mu$ l 10 $\times$ LA PCR buffer (manufactured by TAKARA SHUZO CO., LTD), each 20 nmol of four kinds of nucleotides (dATP, dCTP, dGTP, dTTP: manufactured by Clontech), and 10ng of phage cloned DNA in a 0.5ml volume of a polymerase chain reaction tube and adding sterile distilled water to total 50  $\mu$ l. Each step of polymerase chain reaction was carried out under the following conditions: The first cycle comprising a denaturing step holding a temperature at 95  $^{\circ}$ C for 1 minute, an annealing step holding a temperature at 55 $^{\circ}$ C for 2 minutes, and an extension step with a DNA polymerase holding a temperature at 72 $^{\circ}$ C for 3 minutes was performed once, and the second cycle comprising a denaturing step holding a temperature at 95 $^{\circ}$ C for 1 minute, an annealing step holding a temperature at 55 $^{\circ}$ C for 1.5 minutes, and an extension step with a polymerase holding a temperature at 72 $^{\circ}$ C for 2 minutes was performed 34 times. After completion of the polymerase chain reaction, the reaction solution was filtered by MicroSpin S-400HR (manufactured by Pharmacia Biotech) to purify the DNA fragments amplified by the polymerase chain reaction.

Next, 2  $\mu$ l of the purified solution of the DNA fragment amplified by the above polymerase chain reaction, 50 ng of linearized pCR2.1 (manufactured by Invitrogen), 1  $\mu$ l of Ligation buffer (manufactured by Invitrogen), and 4 Weiss units of T4 DNA Ligase (manufactured by Invitrogen) were taken into 1.5 ml microtube, a sterile distilled water was added thereto to total 10  $\mu$ l to mix, a DNA ligation reaction was performed by holding a temperature at 14  $^{\circ}$ C for 16 hours to ligate the DNA fragment amplified by the polymerase chain reaction to pCR2.1. After completion of

ligation reaction, a competent cell of *Escherichia coli* strain INV  $\alpha$  F'(manufactured by Invitrogen) was transformed with 1  $\mu$ l of the reaction solution to select a strain which became ampicillin-resistant. Further, plasmid DNA was extracted from the selected strain and they were cut with restriction enzymes and analyzed on an agarose electrophoresis to select a plasmid in which about 1.4kbp DNA fragment amplified by polymerase chain reaction was cloned.

The nucleotide sequence of the DNA fragment harbored by the selected plasmid was determined using Dye terminator cycle sequencing kit (PE Applied Bio Systems) and a DNA sequencer 373S (manufactured by PE Applied Bio Systems). The determined nucleotide sequence was analyzed and found that the cloned 1.4kbp DNA fragment overlap with the DNA fragment obtained in Example 1 by about 1.1kbp and contain about 150bp upstream from translation initiation point.

The above plasmid having about 1.4kbp DNA fragment was cut with a restriction enzyme, EcoRI and HincII (both manufactured by TAKARA SHUZO CO., LTD) to recover about 550bp fragment. On the other hand, the plasmid harbored by the clone obtained in Example 1 was cut with a restriction enzyme, EcoRI and HincII (both manufactured by TAKARA SHUZO CO., LTD) and its 5' terminal was dephosphorylated with calf intestine alkaline phosphatase (manufactured by TAKARA SHUZO CO., LTD).

The aforementioned two DNA fragments were taken in a microtube, and ligated using a DNA ligation kit (manufactured by TAKARA SHUZO CO., LTD). *Escherichia coli* HB101 competent cell (manufactured by TAKARA SHUZO CO., LTD) was transformed with the resultant reaction solution and selected a strain which

became ampicillin-resistant. The plasmid harbored by the selected strain was cut with restriction enzymes EcoRI and XhoI (both manufactured by TAKARA SHUZO CO., LTD), and analyzed on an agarose gel electrophoresis to select a plasmid into which about 1.7kbp DNA fragment is cloned.

The nucleotide sequence of the DNA fragment harbored by the resultant clone was determined using Dye terminator cycle sequencing kit (manufactured by PE Applied Bio Systems) and a DNA sequencer 373S (manufactured by PE Applied Bio Systems). As a result, the nucleotide sequence shown by SEQ ID: No.2 was revealed and the cloned DNA fragment was found to contain a full length structural gene (1431bp) encoding a rat-derived PPO.

#### Example 3 (Analysis of PPO amino acid sequence)

The nucleotide sequence of the rat-derived PPO gene cDNA determined in Example 2 was analyzed to translate the amino acid sequence using genetic analyzing software (GENETYX: manufactured by SDC). As a result, a protein encoded by the cloned rat-derived PPO gene cDNA was found to be composed of 477 amino acid residues and its amino acid sequence was that shown by SEQ ID: No. 1.

#### Example 4 (Construction of a vector for expressing a rat PPO gene in *Escherichia coli*)

An oligonucleotide having the nucleotide sequence shown by SEQ ID: No. 5 and that having the nucleotide sequence shown by SEQ ID: No. 6 were prepared. The oligonucleotides were synthesized using a DNA synthesizer (PE Applied Systems: Model 394 DNA/RNA Synthesizer) and using a solvent for Model 394 DNA/RNA Synthesizer as a solvent (PE Applied Systems) and using as a DNA synthesizing reagent phosphoramidite reagents corresponding to adenine, cytosine, guanine and thymidine

(PE Applied Systems). The synthesized oligonucleotides were purified by an oligonucleotide purifying cartridge (PE Applied Systems: OPC cartridge) and dried under reduced pressure to prepare oligonucleotides.

An polymerase chain reaction was performed using as a template a full length gene cDNA encoding the rat-derived PPO obtained in Example 2 and as a primer an oligonucleotide having the nucleotide sequence shown by SEQ ID: No. 5 and that having the nucleotide sequence shown by SEQ ID: No.6 to amplify the about 1.5kbp DNA fragment encoding PPO. A reaction solution in the polymerase chain reaction was prepared by taking 10pmol of an oligonucleotide having the nucleotide sequence shown by SEQ ID; No.5, 10pmol of an oligonucleotide having the nucleotide sequence shown by SEQ ID: No. 6, 0.5  $\mu$  l of TaKaRa LATaq (manufactured by TAKARA SHUZO CO., LTD), 5.0  $\mu$  l 10 $\times$  LA PCR buffer (manufactured by TAKARA SHUZO CO., LTD), each 20 nmol of four kinds of nucleotides (dATP, dCTP, dGTP and dTTP; manufactured by Clontech), and 10ng of a plasmid containing full length cDNA of the rat PPO obtained in Example 2 in a 0.5ml volume of a polymerase chain reaction tube and adding sterile distilled water to total 50  $\mu$  l. Each step in the polymerase chain reaction was performed under the following conditions: The first cycle comprising a denaturing step holding a temperature at 95 $^{\circ}$ C for 1 minute, an annealing step holding a temperature at 55 $^{\circ}$ C for two minutes, and extension step with a DNA polymerase holding a temperature at 72 $^{\circ}$ C for three minutes was performed once, and the second cycle comprising a denaturing step holding a temperature at 95 $^{\circ}$ C for 1 minute, an annealing step holding a temperature at 55 $^{\circ}$ C for 1.5 minutes, and an



extension step holding a temperature at 72°C for 2 minutes was performed 34 times.

After the polymerase chain reaction, the DNA fragment amplified by the polymerase chain reaction was purified by filtering the reaction solution with MicroSpin S-400HR (manufactured by Pharmacia Biotech). A terminus of this DNA fragment was cut with restriction enzymes SacII and SmaI. On the other hand, pBluescript II SK+ (manufactured by Stratagene) was cut with restriction enzymes SacII and SmaI (both manufactured by TAKARA SHUZO CO., LTD) and the 5' end was dephosphorylated with calf intestine alkaline phosphatase (manufactured by TAKARA SHUZO CO., LTD).

The aforementioned two DNA fragments were taken in a microtube, and ligated using a DNA ligation kit (manufactured by TAKARA SHUZO CO., LTD). A competent cell of *Escherichia coli* JM109 (manufactured by TAKARA SHUZO CO., LTD) was transformed with the resultant reaction solution to select a strain which became ampicillin-resistant and a plasmid DNA was prepared from the ampicillin-resistant strain to obtain a vector for expressing rat PPO gene in *Escherichia coli*.  
Example 5 (Complementation experiment of *Escherichia coli hemG*-deficient strain with rat PPO gene)

The expression vector obtained in Example 4 was introduced into *Escherichia coli* PPO gene (*hemG* locus)-deficient mutant strain BT3, which was spread-inoculated on LB agar medium containing kanamycin at final concentration of 10  $\mu$  g/ml and ampicillin at final concentration of 50  $\mu$  g/ml to culture at 37°C for 2 days. BT3 strain became less proliferative and formed only small colonies, while a strain having

the introduced vector for expressing rat PPO gene formed relatively large colonies on the LB plate.

Example 6 (Test on the ability of a compound to inhibit the rat PPO activity)

An overnight cultured bacterial solution of *Escherichia coli* strain BT3 into which a introduced vector for expressing the rat PPO gene obtained in Example 5 is diluted to turbidity( $OD_{600}$ ) of 1.0. 0.05% medium volume equivalent amount of this diluted bacterial solution is inoculated on YPT liquid medium containing no various test compounds and also is inoculated on YPT liquid medium containing various compounds at a variety of concentrations to culture by shaking at 37°C and turbidity ( $OD_{600}$ ) 16 hours after culturing is measured. The concentration at which 50% growth inhibition by various test compounds is seen is calculated by adopting turbidity of the system having no addition of test compound as 100%. By comparing the concentrations in various test compounds, the PPO inhibiting abilities of test compounds are determined.

Example 7 (Obtaining of *Escherichia coli* for expressing rat PPO gene, into which *lac* repressor gene is introduced)

pBR322 was cut with restriction enzymes HindIII and AvaI (both manufactured by TAKARA SHUZO CO., LTD) to recover the about 1.4kb fragment. Separately, pREP4 was cut with restriction enzymes HindIII and AvaI (both manufactured by TAKARA SHUZO CO., LTD) to recover the about 2.4kb fragment and its 5'end was dephosphorylated with calf intestine alkaline phosphatase (manufactured by TAKARA SHUZO CO., LTD).

The aforementioned two DNA fragments were taken in a microtube and ligated

using a DNA ligation kit (manufactured by TAKARA SHUZO CO., LTD). A competent cell (manufactured by TAKARA SHUZO CO., LTD) of *Escherichia coli* strain JM109 was transformed with the resultant reaction solution to select a strain which became kanamycin and tetracyclin-resistant and a plasmid DNA was prepared from the kanamycin and tetracyclin-resistant strain to obtain a vector for expressing *lac* repressor. The vector was introduced into *Escherichia coli* strain BT3 to select a strain which became kanamycin, tetracyclin and ampicillin-resistant. Further, the expression vector obtained in Example 4 was introduced into the tetracyclin-resistant strain to select a strain which became kanamycin and tetracyclin- and ampicillin-resistant to obtain *Escherichia coli* strain BT3 into which the rat PPO gene and *lac* repressor gene were introduced.

Example 8 (Assaying of the ability of a compound to inhibit the PPO activity using *Escherichia coli* strain BT3 for expressing rat PPO gene, into which *lac* repressor is introduced)

An overnight cultured bacterial solution of *Escherichia coli* strain BT3 for expressing a rat PPO gene, into which *lac* repressor was introduced, obtained in Example 7 was diluted to turbidity ( $OD_{600}$ ) of 0.5. 0.1% medium volume equivalent amount of this diluted bacterial solution was inoculated on YPT liquid medium containing no test compound and also was inoculated on YPT liquid medium containing various test compounds at various concentrations to culture by shaking at 37°C and turbidity ( $OD_{600}$ ) 20 hours after culturing was measured. The concentration at which 50% growth inhibition by a compound having the structure shown in Figure 4 was seen was calculated and found to be 0.11ppm under the condition that the growth rate of the

transformant is 100% in the system of no addition of test compound.

Example 9 (Cloning of *Chlamydomonas reinhardtii* PPO DNA fragment)

*Chlamydomonas reinhardtii* strain CC407 was obtained from *Chlamydomonas* Genetics Center (address: DCMB Group, Department of Botany, Box 91000, Duke University, Durham, NC 27708-1000, USA), and cultured in TAP liquid medium comprising 7mM NH<sub>4</sub>Cl, 0.4mM MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.34mM CaCl<sub>2</sub> · 2H<sub>2</sub>O, 25mM potassium calcium, 0.5mM Tris (pH 7.5), 1ml/L Hutner trace elements, and 1ml/L glacial acetic acid (E.H.Harris, The *Chlamydomonas* Sourcebook, Academic Press, San Diego, 1989, pp576-577) under the light (200  $\mu$  E/m<sup>2</sup>/s<sup>1</sup>) for 5 days to obtain 200ml of culture containing cells at early stationary proliferation stage ( $1.0 \times 10^6$  cells/ml).

The whole RNA was prepared from the cells by performing the manipulation using ISOGEN (Nippon gene) according to the attached manual. Further, poly(A) RNA was fractionated using BioMag mRNA Purification Kit (Perceptive Biosystems) by performing the manipulation according to the attached manual. From the resultant poly(A)RNA, a cDNA was synthesized using Marathon cDNA Amplification Kit (Clontech) by performing the manipulation according to the attached manual and this was used as a template for polymerase chain reaction.

An oligonucleotide having the nucleotide sequence shown by SEQ ID: No. 11 and that having the nucleotide sequence shown by SEQ ID: 12 were prepared as a primer for polymerase chain reaction. The oligonucleotides were synthesized using a DNA synthesizer (PE Applied Systems: Model 394 DNA/RNA Synthesizer) and using as a solvent for synthesis a solvent for Model 394 DNA/RNA Synthesizer (PE Applied

Systems) and using as a reagent for synthesizing DNA phosphoramidite reagents corresponding to adenine, cytosine, guanine and thymine (PE Applied Systems). Synthesized oligonucleotides were purified with oligonucleotide purifying cartridge (PE Applied Systems: OPC cartridge) and dried under reduced pressure to prepare an oligonucleotide.

A polymerase chain reaction was performed by preparing a reaction solution using Advantage cDNA PCR kit (Clontech) according to the attached manual and repeating once a cycle comprising 94°C for 1 minute and 70°C for 4 minutes, four times a cycle comprising 94°C for 10 seconds, then 70°C for 4 minutes, five times a cycle comprising 94°C for 10 seconds, then 68°C for 4 minutes, and 25 times a cycle comprising 94°C for 10 seconds, then 65°C for 5 minutes, and an aliquot of the reaction solution was subjected to agarose gel electrophoresis to confirm that about 2kbp amplified fragment is obtained. Further, the excess primers in the reaction solution were removed by performing the manipulation using MicroSpin S400HR column (Pharmacia Biotech) according to the attached manual and the manipulation was performed using TA Cloning Kit (Invitrogen) according to the attached manual to clone the amplified fragment into pCR2.1 plasmid.

The nucleotide sequence of the DNA fragment harbored by the resultant recombined plasmid was determined using Dye terminator cycle sequencing kit (manufactured by PE Applied Bio Systems) and a DNA sequencer 373S (manufactured by PE Applied Bio Systems). As a result, the nucleotide sequence shown by SEQ ID; No. 10 was revealed and it was found to be full length cDNA encoding the amino acid

sequence shown by SEQ ID: No. 9 by the analysis using Genetic Analyzing software GENETYX (SDC).

Example 10 (Construction of a vector for expressing a *Chlamydomonas reinhardtii* PPO gene in *Escherichia coli*)

An oligonucleotide having the nucleotide sequence shown by SEQ ID: No. 13, in which a restriction enzyme SacI recognition sequence is introduced at an end, and an oligonucleotide having the nucleotide sequence shown by SEQ ID: No. 14, in which a restriction enzyme SalI recognition sequence is introduced at an end were prepared.

The oligonucleotides were synthesized using a DNA synthesizer (PE Applied Systems: Model 394 DNA/RNA Synthesizer) and as a solvent for synthesis a solvent for Model 394 DNA/RNA Synthesizer (PE Applied Systems) and using as a reagent for DNA synthesis a phosphoramidite reagent corresponding to adenine, cytosine, guanine, and thymine (PE Applied Systems). The synthesized oligonucleotides were purified by an oligonucleotide purifying cartridge (PE Applied Systems: OPC cartridge) and dried under reduced pressure to prepare an oligonucleotide.

A polymerase chain reaction was performed using as a template a full length gene cDNA encoding PPO derived from *Chlamydomonas reinhardtii* obtained in Example 9 and as a primer an oligonucleotide having the nucleotide sequence shown by SEQ ID: No. 13 and that having the nucleotide sequence shown by SEQ ID: No. 14 to amplify the about 2kbp DNA fragment encoding PPO. A reaction solution in the polymerase chain reaction was prepared by taking 10pmol of an oligonucleotide having the nucleotide sequence shown by SEQ ID: No. 13, 10pmol of an oligonucleotide having the nucleotide sequence shown by SEQ ID: No. 14, 0.5  $\mu$  l of TaKaRa LATaq

(manufactured by TAKARA SHUZO CO., LTD), 5.0  $\mu$  l of 10 $\times$ LA PCR buffer (manufactured by TAKARA SHUZO CO., LTD), each 20nmol of four kinds of nucleotides (dATP, dCTP, dGTP, dTTP: manufactured by: Clontech), 10ng of a plasmid containing full length cDNA of *Chlamydomonas reinhardtii* PPO obtained in Example 9 in 0.5 ml volume of a polymerase chain reaction tube and adding sterile distilled water to total 50  $\mu$  l. Each step in the polymerase chain reaction was performed under the following conditions; After the first cycle comprising a denaturing step holding a temperature at 95 $^{\circ}$ C for 1 minute, an annealing step holding a temperature at 55 $^{\circ}$ C for 2 minutes, and an extension step with a DNA polymerase holding a temperature at 72 $^{\circ}$ C for 3 minutes was performed once, the second cycle comprising a denaturing step holding a temperature at 95 $^{\circ}$ C for 1 minute, an annealing step holding a temperature at 55 $^{\circ}$ C for 15 minutes, and an extension step holding a temperature at 72 $^{\circ}$ C for 2 minutes was performed 34 times.

After the polymerase chain reaction, the DNA fragment amplified by the polymerase chain reaction was purified by filtering the reaction solution with MicroSpin S-400HR (manufactured by Pharmacia Biotech). The end of this DNA fragment was cut with restriction enzymes SacI and SalI. On the other hand, the plasmid pUC118 (manufactured by TAKARA SHUZO CO., LTD) was cut with restriction enzymes SacI and SalI (both manufactured by TAKARA SHUZO CO., LTD) and 5'end was dephosphorylated with calf intestine alkaline phosphatase (manufactured by TAKARA SHUZO CO., LTD).

The aforementioned two DNA fragments were taken in a microtube and ligated

using a DNA ligation kit (manufactured by TAKARA SHUZO CO., LTD). A competent cell (manufactured by TAKARA SHUZO CO., LTD) of *Escherichia coli* JM109 was transformed with the resultant reaction solution to select a strain which became ampicillin-resistant and a plasmid DNA was prepared from the ampicillin-resistant strain to obtain a vector for expressing *Chlamydomonas reinhardtii* PPO gene.

Example 11 (Complementation experiment of *Escherichia coli* *hemG*-deficient strain with *Chlamydomonas reinhardtii* PPO gene)

The expression vector obtained in Example 10 was introduced into PPO gene (*hemG* locus)-deficient mutant *Escherichia coli* strain BT3 described in F.Yamamoto, H.Inokuchi, H.Ozeki, (1988) Japanese Journal of Genetics, vol.63, pp237 to 249, which was spread-inoculated on LB agar medium containing kanamycin to the final concentration of 10  $\mu$  g/ml and ampicillin to the final concentration of 50  $\mu$  g/ml to culture at 37°C for 2 days. *Escherichia coli* BT3 strain became less proliferative and formed small colonies, while a strain having introduced vector for expressing *Chlamydomonas reinhardtii* PPO gene formed relatively large colonies on the LB plate.

Example 12 (Construction of a PPO gene expressing vector for direct introduction)

In order to express a rat-derived PPO gene in a plant cell, a PPO gene expressing vector for directly introducing a plant is constructed.

An oligonucleotide having the nucleotide sequence shown by SEQ ID: No. 7 and that having the nucleotide sequence shown by SEQ ID: No.8 are prepared. The oligonucleotides are synthesized using a DNA synthesizer (PE Applied Systems: Model 394 DNA/RNA Synthesizer) and using as a solvent for DNA synthesis a solvent for Model 394 DMA/RNA Synthesizer (PE Applied Systems) and as a reagent for synthesis



phosphoramidite reagents corresponding to adenine, cytosine, guanine and thymine (PE Applied Systems). The synthesized oligonucleotides are purified by an oligonucleotide purifying cartridge (PE Applied Systems: OPC cartridge) and dried under reduced pressure to prepare an oligonucleotide.

A polymerase chain reaction is performed using as a template a full length cDNA of the rat-derived PPO obtained in Example 2, and using as a primer an oligonucleotide having the nucleotide sequence shown in SEQ ID:No. 7 and that having the nucleotide sequence shown in SEQ ID:No. 8 to amplify the about 1.5kbp DNA fragment encoding the rat PPO. The polymerase chain reaction is performed by adding 10pmol of an oligonucleotide having the nucleotide sequence shown in SEQ ID:No. 7, 10pmol of an oligonucleotide having the nucleotide sequence shown in SEQ ID:No. 8, 0.5  $\mu$  l of Advantage KlenTaq Polymerase Mix (manufactured by Clontech), 2.5  $\mu$  l of 10 $\times$  KlenTaq PCR reaction buffer (manufactured by Clontech), each 5nmol of four kinds of nucleotides (dATP, dCTP, dGTP, dTTP: manufactured by Clontech), and 10ng of the full length of cDNA of the rat-derived PPO obtained in Example 2 in a 0.2ml volume of a polymerase chain reaction tube to total amount of 25  $\mu$  l. Each step in the polymerase chain reaction is performed under the following conditions: After the first cycle comprising a denaturing step holding a temperature at 94 $^{\circ}$ C for 1 minute, and an annealing step and extension step with a DNA polymerase holding a temperature at 65 $^{\circ}$ C for 4 minutes is performed once, the second cycle comprising a denaturing step holding a temperature at 94 $^{\circ}$ C for 30 seconds, and an annealing step and extension step with a DNA polymerase holding a temperature at 65 $^{\circ}$ C for 4 minutes is performed 15

times. After completion of the polymerase chain reaction, the DNA fragment amplified in the polymerase chain reaction is purified by filtering the reaction solution with MicroSpin S-400HR (manufactured by Pharmacia Biotech). After the end of this DNA fragment is made blunt with a DNA blunting kit (manufactured by TAKARA SHUZO CO., LTD), a phosphate group is added to the 5'end with T4 polynucleotide kinase (manufactured by TAKARA SHUZO CO., LTD).

On the other hand, after the GUS expression vector pBI221 derived from pUC19 (manufactured by Clontech) is cut with restriction enzymes SmaI and SacI (both manufactured by TAKARA SHUZO CO., LTD) to recover the 2.8kbp DNA fragment from which the GUS structural gene has been removed and its end is made blunt using a DNA blunting kit (manufactured by TAKARA SHUZO CO., LTD), the DNA fragment is dephosphorylated with bacterial alkaline phosphatase (manufactured by TAKARA SHUZO CO., LTD).

The aforementioned two DNA fragments are taken in a microtube and ligated using a DNA ligation kit (manufactured by TAKARA SHUZO CO., LTD). A competent cell of *Escherichia coli* strain HB101 (manufactured by TAKARA SHUZO CO., LTD) is transformed with the resultant reaction solution to select a strain which has become ampicillin-resistant. Further, a plasmid in which a coding region for the rat-derived PPO is inserted in a forward direction relative to the cauliflower mosaic virus-derived 35S promoter and the nopaline synthase-derived terminator is selected from plasmids contained in the selected strain, to obtain a rat PPO gene expression vector for directly introducing into a plant.

Example 13 (Construction of a PPO gene expression vector for indirect introduction)

In order to express a rat-derived PPO gene to in a plant cell, a PPO gene expression vector for indirect introduction into a plant is constructed.

An oligonucleotide having the nucleotide sequence shown by SEQ ID: No.7 and an oligonucleotide having the nucleotide sequence shown by SEQ ID: No.8 are prepared. The oligonucleotides are synthesized using a DNA synthesizer (PE Applied Systems: Model 394 DNA/RNA Synthesizer), using a solvent for synthesis a solvent for Model 394 DNA/RNA Synthesizer (PE Applied Systems) and using as a reagent for DNA synthesis phosphoramidite reagents corresponding to adenine, cytosine, guanine and thymine (PE Applied Systems). After the synthesized oligonucleotides are purified with an oligonucleotide purifying cartridge (PE Applied Systems: OPC cartridge), they are dried under reduced pressure to prepare the oligonucleotides.

A polymerase chain reaction is performed using as a template the full length cDNA for the rat-derived PPO obtained in Example 2, and using an oligonucleotide having the nucleotide sequence shown in SEQ ID: No. 7 and that having the base sequence shown in SEQ ID: No. 8 to amplify the about 1.5kbp DNA fragment encoding the rat-derived PPO. The polymerase chain reaction is performed by adding 10pmol of an oligonucleotide having the nucleotide sequence shown by SEQ ID: No. 7, 10pmol of an oligonucleotide having the nucleotide sequence shown by SEQ ID: No. 8, 0.5  $\mu$  l of Advantage KlenTaq Polymerase Mix (manufactured by Clontech), 2.5  $\mu$  l 10 $\times$  KlenTaq PCR reaction buffer (manufactured by Clontech), each 5n mol of four kinds of nucleotides (dATP, dCTP, dGTP, dTTP, manufactured by Clontech), and 10ng of a DNA containing the full length cDNA for the rat-derived PPO obtained in Example 2 to

0.2  $\mu$  l volume of a polymerase chain reaction tube to total volume of 25  $\mu$  l. Each step of the polymerase chain reaction is performed as follows: After the first cycle comprising a denaturing step holding a temperature at 94°C for 1 minute, and an annealing step and an extension step with a DNA polymerase holding a temperature at 65°C for 4 minutes is performed once, the second step comprising a denaturing step holding a temperature at 94°C for 30 seconds, an annealing step and an extension step with a DNA polymerase holding a temperature at 65°C for 4 minutes is performed 15 times. After completion of the polymerase chain reaction, the DNA fragment amplified by polymerase chain reaction is purified by filtering the reaction solution with MicroSpin S-400HR (manufactured by Pharmacia Biotech). After the end of this DNA fragment is made blunt with a DNA blunting kit (manufactured by TAKARA SHUZO CO., LTD), a phosphate group is added to the 5'end with the T4 polynucleotide kinase (manufactured by TAKARA SHUZO CO., LTD).

On the other hand, after the pBIN19-derived GUS expressing binary vector pBI121 (manufactured by Clontech) is cut with restriction enzymes SmaI and SacI (both manufactured by TAKARA SHUZO CO., LTD) to recover the DNA fragment from which the GUS structural gene has been removed and its end is made blunt using a DNA blunting kit (manufactured by TAKARA SHUZO CO., LTD), it is dephosphorylated with a bacterial alkaline phosphatase (manufactured by TAKARA SHUZO CO., LTD).

The aforementioned two DNA fragments are taken in a microtube and ligated using a DNA ligation kit (manufactured by TAKARA SHUZO CO., LTD). A

competent cell of *Escherichia coli* strain HB101 (manufactured by TAKARA SHUZO CO., LTD) is transformed with the resultant reaction solution to select a strain which has become kanamycin-resistant. Further, a plasmid in which a coding region for a rat PPO is inserted in a forward direction relative to the cauliflower mosaic virus-derived 35S promoter and the nopaline synthase-derived terminator from plasmids contained in the selected strain, to obtain the rat PPO gene expression vector for indirect introduction into a plant.

Example 14 (Construction of a vector for expressing a *Chlamydomonas reinhardtii* PPO gene)

In order to express a *Chlamydomonas reinhardtii*-derived PPO in a plant cell, a PPO gene expression vector for direct introduction into a plant is constructed.

The pCR2.1 plasmid harboring the amplified fragment of the cDNA for the *Chlamydomonas reinhardtii*-derived PPO gene obtained in Example 9 is digested with restriction enzymes NotI and SpeI (both manufactured by TAKARA SHUZO CO., LTD) to obtain the about 2kbp *Chlamydomonas reinhardtii*-derived PPO gene cDNA fragment having cohesive ends for NotI and SpeI at its end. On the other hand, pBluescriptII KS+ (manufactured by Stratagene) is cut with restriction enzymes NotI and SpeI (both manufactured by TAKARA SHUZO CO., LTD) and the 5'end is dephosphorylated with calf intestine alkaline phosphatase (manufactured by TAKARA SHUZO CO., LTD). The two DNA fragments are taken in a microtube and ligated using a DNA ligation kit (manufactured by TAKARA SHUZO CO., LTD). A competent cell of *Escherichia coli* strain HB101 (manufactured by TAKARA SHUZO CO., LTD) is transformed with the resultant reaction solution to select a strain which

has become ampicillin-resistant. Further, a plasmid contained in the selected strain is selected to obtain a clone in which the *Chlamydomonas reinhardtii*-derived PPO gene cDNA is inserted into the plasmid pBluescriptII KS+.

The resultant plasmid is digested with restriction enzymes BamHI and SacI (both manufactured by TAKARA SHUZO CO., LTD) to obtain the about 2kbp *Chlamydomonas reinhardtii*-derived PPO gene cDNA fragment having cohesive ends for BamHI and SacI at its ends. On the other hand, pBI221 and pBI121 (both manufactured by Clontech) are cut with restriction enzymes BamHI and SacI (both manufactured by TAKARA SHUZO CO., LTD), respectively, to obtain a vector fragment from which a  $\beta$ -glucuronidase gene has been removed and the 5'end is dephosphorylated with the calf intestine alkaline phosphatase (manufactured by TAKARA SHUZO CO., LTD). The *Chlamydomonas reinhardtii*-derived PPO gene cDNA fragment and the two DNA fragments of the vector fragment of pBI221 or pBI121 are taken in a microtube and ligated using a DNA ligation kit (manufactured by TAKARA SHUZO CO., LTD). A competent cell of *Escherichia coli* strain HB101 (manufactured by TAKARA SHUZO CO., LTD) is transformed with the resultant reaction solution and a strain which has become ampicillin-resistant, in the case of ligation with the pBI221 vector fragment and, a plasmid which has become kanamycin-resistant, in the case of ligation with pBI121 vector fragment are selected. Further, a plasmid contained in the selected strain is select to obtain a plasmid for direct introduction in which the *Chlamydomonas reinhardtii*-derived PPO gene cDNA fragment is inserted into the pBI221 vector fragment or a plasmid for indirect introduction in which the *Chlamydomonas reinhardtii*-derived PPO gene cDNA

fragment is inserted into the pBI121 vector fragment.

Example 15 (Production of a transformed plant in which a rat or *Chlamydomonas reinhardtii*-derived PPO expression vector is introduced)

The rat PPO gene expression vector for indirect introduction or the *Chlamydomonas reinhardtii* PPO gene expression vector for indirect induction obtained in Example 13 or Example 14 is transferred into *Agrobacterium tumefaciens* LBA4404 by a binary vector method (manufactured by Clontech: GUS Gene Fusion System). A sterile-cultured tobacco leaf is infected with this bacterial strain according to Plant Gene Manipulation Manual (author: Uchimiya, Kodansha Scientific, 1990) to obtain a transformed tobacco. Similarly, a sterile-cultured carrot seedling petiole is infected therewith according to a method described in N.Pawlicki et al., (1992) Plant Cell, Tissue and Organ Culture, vol.31, pp.129 to 139 to obtain a transformed carrot.

Similarly, a sterile-cultured pea seedling epicotyl or cotyledon is infected therewith according to a method described in J.Puonti-Kaerlas et al., (1990) Theoretical and Applied Genetics, vol.80, pp.246 to 252 to obtain a transformed pea.

Further, the rat PPO gene expression vector for direct introduction or *Chlamydomonas reinhardtii* PPO gene expression vector for direct introduction obtained in Example 12 or Example 15 is introduced into soybean adventitious embryo with a particle gun according to a method described in JP-A-3-291501 (laid-opened) to obtain a transformed soybean. Similarly, it is introduced into a sterile-cultured rice immature scutellum with a particle gun according to a method described in Ikushugakkaishi, vol.44, suppl.No.1, pp.66, (1994)(author: Shimada, et al.) to obtain a transformed rice. Similarly, it is introduced into a sterile-cultured wheat immature

scutellum with a particle gun according to the conventional method described in Ikushugakkaishi, vol.44, suppl. No.1, pp.57 (1995) (author: Takumi, et al.) to obtain a transformed wheat. Similarly, it is introduced into a sterile-cultured barley immature scutellum with a particle gun according to a method described in Ikushugakkaishi, vol.44, suppl. No.1, pp.67 (1995)(author: Hagio, et al.) to obtain a transformed barley. Similarly, it is introduced into a corn adventitious embryo with a particle gun according to a method described in M.E. Fromm et al., (1990) BIO/TECHNOLOGY, vol.8, pp.833 to 839 to obtain a transformed corn.

The present invention makes it possible to provide a method for evaluating the ability of a compound to inhibit the PPO activity with simplicity.

#### Brief explanation of the sequence

##### 1. SEQ ID: No.1

Shows the amino acid sequence of a mitochondrial-type PPO encoded in a cDNA for a rat-derived PPO gene.

##### 2. SEQ ID: No.2

Shows the nucleotide sequence of a cDNA clone for a rat-derived PPO gene.

##### 3. SEQ ID: No.3

Shows the nucleotide sequence of an oligonucleotide used for amplifying a DNA fragment containing a partial nucleotide sequence of a rat-derived PPO gene.

##### 4. SEQ ID: No.4

Shows the nucleotide sequence of an oligonucleotide used for amplifying a DNA fragment containing a partial nucleotide sequence of a rat-derived PPO gene.



5. SEQ ID: No. 5

Shows the nucleotide sequence of a primer used for constructing a vector expressing a rat-derived PPO gene in *Escherichia coli*.

6. SEQ ID: No. 6

Shows the nucleotide sequence of a primer used for constructing a vector expressing a rat-derived PPO gene in *Escherichia coli*.

7. SEQ ID: No. 7

Shows the nucleotide sequence of a primer used for constructing a rat-derived PPO gene expression vector for direct introduction and a rat-derived PPO expression vector for indirect introduction.

8. SEQ ID: No.8

Shows the nucleotide sequence of a primer used for constructing a rat-derived PPO gene expression vector for direct introduction and a rat-derived PPO expression vector for indirect introduction.

9. SEQ ID: No. 9

Shows the amino acid sequence of PPO encoded in a cDNA for *Chlamydomonas reinhardtii*-derived PPO gene.

10. SEQ ID: No.10

Shows the nucleotide sequence of a cDNA clone for *Chlamydomonas reinhardtii*-derived PPO gene.

11. SEQ ID: No.11

Shows the nucleotide sequence of an oligonucleotide used for amplifying a DNA fragment containing a *Chlamydomonas reinhardtii*-derived PPO gene.

12. SEQ ID: No.12

Shows the nucleotide sequence of an oligonucleotide used for amplifying a DNA fragment containing a *Chlamydomonas reinhardtii*-derived PPO gene.

13. SEQ ID: No. 13

Shows the nucleotide sequence of a primer used for constructing a vector expressing a *Chlamydomonas reinhardtii*-derived PPO gene in *Escherichia coli*.

14. SEQ ID: No.14

Shows the nucleotide sequence of a primer used for constructing a vector for expressing a *Chlamydomonas reinhardtii*-derived PPO gene in *Escherichia coli*.

00229130.03163260

# SEQUENCE LISTING

<110> Yasutaka SHIMOKAWATOKO et al.; Sumitomo Chemical Company Limited

<120> A method for evaluating the ability of a compound to inhibit the  
protoporphyrinogen oxidase activity

<130> P148946

<150> JP 10/099619

<151> 1998-04-10

<160> 14

<210> 1

<211> 477

<212> PRT

<213> Rattus norvegicus

<400> 1

```

Met Ala Arg Thr Val Ile Val Leu Gly Gly Gly Ile Ser Gly Leu Ala
 1             5             10             15
Ala Ser Tyr His Leu Thr Arg Ser Pro Ser Pro Pro Lys Val Ile Leu
      20             25             30
Val Glu Gly Ser Lys Arg Leu Gly Gly Trp Ile Arg Ser Val Arg Gly
      35             40             45
Ser Asp Gly Ala Ile Phe Glu Leu Gly Pro Arg Gly Ile Arg Pro Ala
      50             55             60
Gly Ala Leu Gly Ala Arg Thr Leu Leu Leu Val Ser Glu Leu Gly Leu
      65             70             75             80
Glu Ser Glu Val Leu Pro Val Arg Gly Asp His Pro Ala Ala Gln Asn
      85             90             95
Arg Phe Leu Tyr Val Gly Gly Ala Leu His Pro Leu Pro Ser Gly Leu
      100            105            110
Arg Gly Leu Leu Arg Pro Ser Pro Pro Phe Ser Lys Pro Leu Phe Trp
      115            120            125
Ala Gly Leu Arg Glu Leu Thr Lys Pro Arg Gly Lys Glu Pro Asp Glu
      130            135            140
Thr Val His Ser Phe Ala Gln Arg Arg Leu Gly Pro Glu Val Ala Ser
      145            150            155            160
Leu Ala Met Asp Ser Leu Cys Arg Gly Val Phe Ala Gly Asn Ser Gln
      165            170            175

```

Glu	Leu	Ser	Ile	Arg	Ser	Cys	Phe	Pro	Ser	Leu	Phe	Gln	Ala	Glu	Gln
			180					185					190		
Thr	His	Gly	Ser	Met	Leu	Leu	Gly	Leu	Leu	Leu	Gly	Ala	Gly	Gln	Thr
		195					200					205			
Pro	Gln	Pro	Asn	Ser	Ser	Leu	Ile	Arg	Gln	Ala	Arg	Ala	Glu	Arg	Trp
	210					215					220				
Ser	Gln	Trp	Ser	Leu	Arg	Gly	Gly	Leu	Glu	Met	Leu	Pro	Gln	Ala	Leu
225					230					235					240
His	Asn	Tyr	Leu	Thr	Ser	Lys	Gly	Val	Thr	Ile	Leu	Ser	Gly	Gln	Pro
				245					250					255	
Ala	Cys	Gly	Leu	Ser	Leu	Gln	Pro	Glu	Gly	His	Trp	Lys	Val	Ser	Leu
			260					265					270		
Gly	Asp	Ser	Ser	Leu	Glu	Ala	Asp	His	Ile	Ile	Ser	Thr	Ile	Pro	Ala
		275					280						285		
Ser	Val	Leu	Ser	Lys	Leu	Leu	Pro	Ala	Glu	Ala	Ala	Pro	Leu	Ala	His
	290					295					300				
Ile	Leu	Ser	Thr	Ile	Gln	Ala	Val	Ser	Val	Ala	Val	Val	Asn	Leu	Gln
305					310					315					320
Tyr	Lys	Gly	Ala	Cys	Leu	Pro	Val	Gln	Gly	Phe	Gly	His	Leu	Val	Pro
				325					330					335	
Ser	Ser	Glu	Asp	Pro	Thr	Val	Leu	Gly	Ile	Val	Tyr	Asp	Ser	Val	Ala
			340					345					350		
Phe	Pro	Glu	Gln	Asp	Gly	Asn	Pro	Pro	Gly	Leu	Arg	Leu	Thr	Val	Met
		355					360					365			
Leu	Gly	Gly	Tyr	Trp	Leu	Gln	Lys	Leu	Lys	Ala	Asn	Gly	His	Glu	Leu
	370					375					380				
Ser	Pro	Glu	Leu	Phe	Gln	Arg	Ala	Ala	Gln	Glu	Ala	Ala	Ala	Thr	Gln
385					390					395					400
Leu	Gly	Leu	Lys	Glu	Gln	Pro	Ser	His	Cys	Leu	Val	His	Leu	His	Lys
				405					410					415	
Asn	Cys	Ile	Pro	Gln	Tyr	Thr	Leu	Gly	His	Trp	Gln	Lys	Leu	Asp	Ser
			420					425					430		
Ala	Leu	Gln	Phe	Leu	Thr	Ala	Gln	Arg	Leu	Pro	Leu	Thr	Leu	Ala	Gly
		435					440					445			
Ala	Ser	Tyr	Glu	Gly	Val	Ala	Val	Asn	Asp	Cys	Ile	Glu	Ser	Gly	Arg
	450					455					460				
Gln	Ala	Ala	Ile	Ala	Val	Leu	Gly	Thr	Glu	Ser	Asn	Ser			
465					470					475					

 $\langle 210 \rangle_2$

<212> DNA

<213> Rattus norvegicus

<220>

<222> (143)...(1576)

<400> 1

CGTACACGCG CGTTTTGCAT TAGTTGCTCA TTAATCAGTA AGTGCCCAGA GGTGGGGTAC 60  
GGGACCCGTG GGGTTTCTGC AGTTGTAAAG CAGGGTGCCT CCCGTTCTCC TGGGGTATCT 120  
CGACTTTCCC CCAGGCCTTA CG ATG GCC CGG ACT GTG ATA GTG CTT GGC GGA 172  
Met Ala Arg Thr Val Ile Val Leu Gly Gly  
1 5 10  
GGT ATC AGC GGA TTG GCC GCA AGT TAT CAT CTG ACC CGA AGC CCC AGT 220  
Gly Ile Ser Gly Leu Ala Ala Ser Tyr His Leu Thr Arg Ser Pro Ser  
15 20 25  
CCT CCT AAG GTG ATC TTA GTG GAG GGC AGC AAA CGT TTG GGA GGC TGG 268  
Pro Pro Lys Val Ile Leu Val Glu Gly Ser Lys Arg Leu Gly Gly Trp  
30 35 40  
ATC CGT TCA GTC CGA GGA TCA GAT GGT GCG ATC TTT GAA CTT GGA CCT 316  
Ile Arg Ser Val Arg Gly Ser Asp Gly Ala Ile Phe Glu Leu Gly Pro  
45 50 55  
CGA GGA ATT AGG CCG GCT GGA GCC CTG GGA GCC CGG ACC CTG CTC CTG 364  
Arg Gly Ile Arg Pro Ala Gly Ala Leu Gly Ala Arg Thr Leu Leu Leu  
60 65 70  
GTT TCT GAA CTT GGC TTG GAA TCC GAA GTC TTG CCT GTC CGA GGG GAT 412  
Val Ser Glu Leu Gly Leu Glu Ser Glu Val Leu Pro Val Arg Gly Asp  
75 80 85 90  
CAT CCA GCT GCC CAG AAC CGG TTC CTG TAT GTA GGC GGT GCC CTG CAC 460  
His Pro Ala Ala Gln Asn Arg Phe Leu Tyr Val Gly Gly Ala Leu His  
95 100 105  
CCC CTA CCC TCT GGC CTC AGG GGG CTA CTT CGT CCT TCA CCC CCC TTC 508  
Pro Leu Pro Ser Gly Leu Arg Gly Leu Leu Arg Pro Ser Pro Pro Phe  
110 115 120  
TCA AAA CCT CTA TTT TGG GCT GGA CTG AGG GAG TTG ACG AAG CCC AGG 556  
Ser Lys Pro Leu Phe Trp Ala Gly Leu Arg Glu Leu Thr Lys Pro Arg  
125 130 135  
GGC AAA GAG CCT GAT GAG ACT GTG CAC AGT TTT GCC CAG CGC CGC CTT 604  
Gly Lys Glu Pro Asp Glu Thr Val His Ser Phe Ala Gln Arg Arg Leu  
140 145 150  
GGA CCT GAG GTG GCG TCT CTG GCT ATG GAC AGC CTT TGC AGA GGA GTG 652



GCC AAT GGC CAT GAA TTG TCT CCA GAG CTA TTC CAA CGA GCA GCA CAG 1324  
 Ala Asn Gly His Glu Leu Ser Pro Glu Leu Phe Gln Arg Ala Ala Gln  
 380 385 390  
 GAA GCG GCT GCC ACA CAG TTA GGA CTG AAA GAG CAA CCA AGC CAT TGC 1372  
 Glu Ala Ala Ala Thr Gln Leu Gly Leu Lys Glu Gln Pro Ser His Cys  
 395 400 405 410  
 TTG GTC CAT CTA CAC AAA AAC TGT ATC CCT CAG TAT ACA CTA GGC CAC 1420  
 Leu Val His Leu His Lys Asn Cys Ile Pro Gln Tyr Thr Leu Gly His  
 415 420 425  
 TGG CAA AAA CTA GAC TCA GCT CTG CAA TTC CTG ACG GCC CAG AGG TTG 1468  
 Trp Gln Lys Leu Asp Ser Ala Leu Gln Phe Leu Thr Ala Gln Arg Leu  
 430 435 440  
 CCC CTG ACT TTG GCT GGG GCC TCC TAT GAG GGG GTA GCT GTC AAT GAC 1516  
 Pro Leu Thr Leu Ala Gly Ala Ser Tyr Glu Gly Val Ala Val Asn Asp  
 445 450 455  
 TGT ATA GAG AGT GGG CGC CAG GCA GCA ATT GCT GTC CTG GGC ACA GAA 1564  
 Cys Ile Glu Ser Gly Arg Gln Ala Ala Ile Ala Val Leu Gly Thr Glu  
 460 465 470  
 TCG AAC AGC TGA CCCCCACTCT CCTACTCATG AAAGTAAAAG TTGATGGAGC 1614  
 Ser Asn Ser  
 475  
 TTGAAAAAAAA AAAAAAAAAA AA  
 1636

<210> 3  
 <211> 28  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Designed oligonucleotide primer used for amplifying a DNA fragment  
 containing a partial nucleotide sequence of a rat-derived PPO gene

<400> 3

TTTGCAGAGG AGTGTGCTTGGCT GGCAACAG 28

<210> 4  
 <211> 29  
 <212> DNA  
 <213> Artificial Sequence

<220>

<223> Designed oligonucleotide primer used for amplifying a DNA fragment containing a partial nucleotide sequence of a rat-derived PPO gene

<400> 4

AGCCGCTTCC TGTGCTGCTC GTTGAATA 29

<210> 5

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Designed oligonucleotide primer used for constructing a vector expressing a rat-derived PPO gene in *Escherichia coli*

<400> 5

AGGCCTTACC GCGGCCCGGA CTGTG 25

<210> 6

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Designed oligonucleotide primer used for constructing a vector expressing a rat-derived PPO gene in *Escherichia coli*

<400> 6

TAGGAGAGCC CGGGTCAGAT GTTCG 25

<210> 7

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Designed oligonucleotide primer used for constructing a rat-derived PPO gene expression vector for direct introduction and a rat-derived PPO expression vector for indirect introduction



<400> 7

ATGGCCCGGA CTGTGATAGT GCTTG 25

<210> 8

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Designed oligonucleotide primer used for constructing a rat-derived PPO gene expression vector for direct introduction and a rat-derived PPO expression vector for indirect introduction

<400> 8

TTCATGAGTA GGAGAGTGGG GGTCA 25

<210> 9

<211> 563

<212> PRT

<213> *Chlamydomonas reinhardtii* CC-407

<400> 9

Met	Met	Leu	Thr	Gln	Thr	Pro	Gly	Thr	Ala	Thr	Ala	Ser	Ser	Arg	Arg
1				5					10					15	
Ser	Gln	Ile	Arg	Ser	Ala	Ala	His	Val	Ser	Ala	Lys	Val	Ala	Pro	Arg
			20					25					30		
Pro	Thr	Pro	Phe	Ser	Val	Ala	Ser	Pro	Ala	Thr	Ala	Ala	Ser	Pro	Ala
			35					40				45			
Thr	Ala	Ala	Ala	Arg	Arg	Thr	Leu	His	Arg	Thr	Ala	Ala	Ala	Ala	Thr
			50				55				60				
Gly	Ala	Pro	Thr	Ala	Ser	Gly	Ala	Gly	Val	Ala	Lys	Thr	Leu	Asp	Asn
65					70					75				80	
Val	Tyr	Asp	Val	Ile	Val	Val	Gly	Gly	Gly	Leu	Ser	Gly	Leu	Val	Thr
				85					90				95		
Gly	Gln	Ala	Leu	Ala	Ala	Gln	His	Lys	Ile	Gln	Asn	Phe	Leu	Val	Thr
			100					105					110		
Glu	Ala	Arg	Glu	Arg	Val	Gly	Gly	Asn	Ile	Thr	Ser	Met	Ser	Gly	Asp
			115					120					125		
Gly	Tyr	Val	Trp	Glu	Glu	Gly	Pro	Asn	Ser	Phe	Gln	Pro	Asn	Asp	Ser

130		135		140
Met Leu Gln Ile Ala Val Asp Ser Gly Cys Glu Lys Asp Leu Val Phe				
145		150		155
Gly Asp Pro Thr Ala Pro Arg Phe Val Trp Trp Glu Gly Lys Leu Arg				
	165		170	175
Pro Val Pro Ser Gly Leu Asp Ala Phe Thr Phe Asp Leu Met Ser Ile				
	180		185	190
Pro Gly Lys Ile Arg Ala Gly Leu Gly Ala Ile Gly Leu Ile Asn Gly				
	195		200	205
Ala Met Pro Ser Phe Glu Glu Ser Val Glu Gln Phe Ile Arg Arg Asn				
	210		215	220
Leu Gly Asp Glu Val Phe Phe Arg Leu Ile Glu Pro Phe Cys Ser Gly				
225		230		235
Val Tyr Ala Gly Asp Pro Ser Lys Leu Ser Met Lys Ala Ala Phe Asn				
	245		250	255
Arg Ile Trp Ile Leu Glu Lys Asn Gly Gly Ser Leu Val Gly Gly Ala				
	260		265	270
Ile Lys Leu Phe Gln Glu Arg Gln Ser Asn Pro Ala Pro Pro Arg Asp				
	275		280	285
Pro Arg Leu Pro Pro Lys Pro Lys Gly Gln Thr Val Gly Ser Phe Arg				
	290		295	300
Lys Gly Leu Lys Met Leu Pro Asp Ala Ile Glu Arg Asn Ile Pro Asp				
305		310		315
Lys Ile Arg Val Asn Trp Lys Leu Val Ser Leu Gly Arg Glu Ala Asp				
	325		330	335
Gly Arg Tyr Gly Leu Val Tyr Asp Thr Pro Glu Gly Arg Val Lys Val				
	340		345	350
Phe Ala Arg Ala Val Ala Leu Thr Ala Pro Ser Tyr Val Val Ala Asp				
	355		360	365
Leu Val Lys Glu Gln Ala Pro Ala Ala Glu Ala Leu Gly Ser Phe				
	370		375	380
Asp Tyr Pro Pro Val Gly Ala Val Thr Leu Ser Tyr Pro Leu Ser Ala				
385		390		395
Val Arg Glu Glu Arg Lys Ala Ser Asp Gly Ser Val Pro Gly Phe Gly				
	405		410	415
Gln Leu His Pro Arg Thr Gln Gly Ile Thr Thr Leu Gly Thr Ile Tyr				
	420		425	430
Ser Ser Ser Leu Phe Pro Gly Arg Ala Pro Glu Gly His Met Leu Leu				
	435		440	445
Leu Asn Tyr Ile Gly Gly Thr Thr Asn Arg Gly Ile Val Asn Gln Thr				
450		455		460

Thr Glu Gln Leu Val Glu Gln Val Asp Lys Asp Leu Arg Asn Met Val  
 465 470 475 480  
 Ile Lys Pro Asp Ala Pro Lys Pro Arg Val Val Gly Val Arg Val Trp  
 485 490 495  
 Pro Arg Ala Ile Pro Gln Phe Asn Leu Gly His Leu Glu Gln Leu Asp  
 500 505 510  
 Lys Ala Arg Lys Ala Leu Asp Ala Ala Gly Leu Gln Gly Val His Leu  
 515 520 525  
 Gly Gly Asn Tyr Val Ser Gly Val Ala Leu Gly Lys Val Val Glu His  
 530 535 540  
 Gly Tyr Glu Ser Ala Ala Asn Leu Ala Lys Ser Val Ser Lys Ala Ala  
 545 550 555 560  
 Val Lys Ala  
 563

<210> 10

<211> 1838

<212> DNA

<213> *Chlamydomonas reinhardtii* CC-407

<220>

<222> (2)...(1793)

<400> 10

A ATG ATG TTG ACC CAG ACT CCT GGG ACC GCC ACG GCT TCT AGC CGG	46
Met Met Leu Thr Gln Thr Pro Gly Thr Ala Thr Ala Ser Ser Arg	
1 5 10 15	
CGG TCG CAG ATC CGC TCG GCT GCG CAC GTC TCC GCC AAG GTC GCG CCT	94
Arg Ser Gln Ile Arg Ser Ala Ala His Val Ser Ala Lys Val Ala Pro	
20 25 30	
CGG CCC ACG CCA TTC TCG GTC GCG AGC CCC GCG ACC GCT GCG AGC CCC	142
Arg Pro Thr Pro Phe Ser Val Ala Ser Pro Ala Thr Ala Ala Ser Pro	
35 40 45	
GCG ACC GCG GCG GCC CGC CGC ACA CTC CAC CGC ACT GCT GCG GCG GCC	190
Ala Thr Ala Ala Ala Arg Arg Thr Leu His Arg Thr Ala Ala Ala Ala	
50 55 60	
ACT GGT GCT CCC ACG GCG TCC GGA GCC GGC GTC GCC AAG ACG CTC GAC	238
Thr Gly Ala Pro Thr Ala Ser Gly Ala Gly Val Ala Lys Thr Leu Asp	
65 70 75	
AAT GTG TAT GAC GTG ATC GTG GTC GGT GGA GGT CTC TCG GGC CTG GTG	286

Asn 80	Val	Tyr	Asp	Val	Ile 85	Val	Val	Gly	Gly	Gly	Leu	Ser	Gly	Leu	Val 95	
ACC	GGC	CAG	GCC	CTG	GCG	GCT	CAG	CAC	AAA	ATT	CAG	AAC	TTC	CTT	GTT	334
Thr	Gly	Gln	Ala	Leu	Ala	Ala	Gln	His	Lys	Ile	Gln	Asn	Phe	Leu	Val	
				100					105					110		
ACG	GAG	GCT	CGC	GAG	CGC	GTC	GGC	GGC	AAC	ATT	ACG	TCC	ATG	TCG	GGC	382
Thr	Glu	Ala	Arg	Glu	Arg	Val	Gly	Gly	Asn	Ile	Thr	Ser	Met	Ser	Gly	
			115					120				125				
GAT	GGC	TAC	GTG	TGG	GAG	GAG	GGC	CCG	AAC	AGC	TTC	CAG	CCC	AAC	GAT	430
Asp	Gly	Tyr	Val	Trp	Glu	Glu	Gly	Pro	Asn	Ser	Phe	Gln	Pro	Asn	Asp	
	130						135				140					
AGC	ATG	CTG	CAG	ATT	GCG	GTG	GAC	TCT	GGC	TGC	GAG	AAG	GAC	CTT	GTG	478
Ser	Met	Leu	Gln	Ile	Ala	Val	Asp	Ser	Gly	Cys	Glu	Lys	Asp	Leu	Val	
	145					150				155						
TTC	GGT	GAC	CCC	ACG	GCT	CCC	CGC	TTC	GTG	TGG	TGG	GAG	GGC	AAG	CTG	526
Phe	Gly	Asp	Pro	Thr	Ala	Pro	Arg	Phe	Val	Trp	Trp	Glu	Gly	Lys	Leu	
160					165				170					175		
CGC	CCC	GTG	CCC	TCG	GGC	CTG	GAC	GCC	TTC	ACC	TTC	GAC	CTC	ATG	TCC	574
Arg	Pro	Val	Pro	Ser	Gly	Leu	Asp	Ala	Phe	Thr	Phe	Asp	Leu	Met	Ser	
			180					185				190				
ATC	CCC	GGC	AAG	ATC	CGC	GCC	GGG	CTG	GGC	GCC	ATC	GGC	CTC	ATC	AAC	622
Ile	Pro	Gly	Lys	Ile	Arg	Ala	Gly	Leu	Gly	Ala	Ile	Gly	Leu	Ile	Asn	
		195					200				205					
GGA	GCC	ATG	CCC	TCC	TTC	GAG	GAG	AGT	GTG	GAG	CAG	TTC	ATC	CGC	CGC	670
Gly	Ala	Met	Pro	Ser	Phe	Glu	Glu	Ser	Val	Glu	Gln	Phe	Ile	Arg	Arg	
	210					215			220							
AAC	CTG	GGC	GAT	GAG	GTG	TTC	TTC	CGC	CTG	ATC	GAG	CCC	TTC	TGC	TCC	718
Asn	Leu	Gly	Asp	Glu	Val	Phe	Phe	Arg	Leu	Ile	Glu	Pro	Phe	Cys	Ser	
	225				230			235								
GGC	GTG	TAC	GCG	GGC	GAC	CCC	TCC	AAG	CTG	TCC	ATG	AAG	GCG	GCC	TTC	766
Gly	Val	Tyr	Ala	Gly	Asp	Pro	Ser	Lys	Leu	Ser	Met	Lys	Ala	Ala	Phe	
240				245				250						255		
AAC	AGG	ATC	TGG	ATT	CTG	GAG	AAG	AAC	GGC	GGC	AGC	CTG	GTG	GGA	GGT	814
Asn	Arg	Ile	Trp	Ile	Leu	Glu	Lys	Asn	Gly	Gly	Ser	Leu	Val	Gly	Gly	
		260				265			270							
GCC	ATC	AAG	CTG	TTC	CAG	GAA	CGC	CAG	TCC	AAC	CCG	GCC	CCG	CCG	CGG	862
Ala	Ile	Lys	Leu	Phe	Gln	Glu	Arg	Gln	Ser	Asn	Pro	Ala	Pro	Pro	Arg	
	275					280			285							
GAC	CCG	CGC	CTG	CCG	CCC	AAG	CCC	AAG	GGC	CAG	ACG	GTG	GGC	TCG	TTC	910
Asp	Pro	Arg	Leu	Pro	Pro	Lys	Pro	Lys	Gly	Gln	Thr	Val	Gly	Ser	Phe	
	290				295			300								

CGC	AAG	GGC	CTG	AAG	ATG	CTG	CCG	GAC	GCC	ATT	GAG	CGC	AAC	ATC	CCC	958
Arg	Lys	Gly	Leu	Lys	Met	Leu	Pro	Asp	Ala	Ile	Glu	Arg	Asn	Ile	Pro	
305			310			315										
GAC	AAG	ATC	CGC	GTG	AAC	TGG	AAG	CTG	GTG	TCT	CTG	GGC	CGC	GAG	GCG	1006
Asp	Lys	Ile	Arg	Val	Asn	Trp	Lys	Leu	Val	Ser	Leu	Gly	Arg	Glu	Ala	
320			325			330			335							
GAC	GGG	CGG	TAC	GGG	CTG	GTG	TAC	GAC	ACG	CCC	GAG	GGC	CGT	GTC	AAG	1054
Asp	Gly	Arg	Tyr	Gly	Leu	Val	Tyr	Asp	Thr	Pro	Glu	Gly	Arg	Val	Lys	
340			345			350										
GTG	TTT	GCC	CGC	GCC	GTG	GCT	CTG	ACC	GCG	CCC	AGC	TAC	GTG	GTG	GCG	1102
Val	Phe	Ala	Arg	Ala	Val	Ala	Leu	Thr	Ala	Pro	Ser	Tyr	Val	Val	Ala	
355			360			365										
GAC	CTG	GTC	AAG	GAG	CAG	GCG	CCC	GCC	GCC	GCC	GAG	GCC	CTG	GGC	TCC	1150
Asp	Leu	Val	Lys	Glu	Gln	Ala	Pro	Ala	Ala	Ala	Glu	Ala	Leu	Gly	Ser	
370			375			380										
TTC	GAC	TAC	CCG	CCG	GTG	GGC	GCC	GTG	ACG	CTG	TCG	TAC	CCG	CTG	AGC	1198
Phe	Asp	Tyr	Pro	Pro	Val	Gly	Ala	Val	Thr	Leu	Ser	Tyr	Pro	Leu	Ser	
385			390			395										
GCC	GTG	CGG	GAG	GAG	CGC	AAG	GCC	TCG	GAC	GGG	TCC	GTG	CCG	GGC	TTC	1246
Ala	Val	Arg	Glu	Glu	Arg	Lys	Ala	Ser	Asp	Gly	Ser	Val	Pro	Gly	Phe	
400			405			410			415							
GGT	CAG	CTG	CAC	CCG	CGC	ACG	CAG	GGC	ATC	ACC	ACT	CTG	GGC	ACC	ATC	1294
Gly	Gln	Leu	His	Pro	Arg	Thr	Gln	Gly	Ile	Thr	Thr	Leu	Gly	Thr	Ile	
420			425			430										
TAC	AGC	TCC	AGC	CTG	TTC	CCC	GGC	CGC	GCG	CCC	GAG	GGC	CAC	ATG	CTG	1342
Tyr	Ser	Ser	Ser	Leu	Phe	Pro	Gly	Arg	Ala	Pro	Glu	Gly	His	Met	Leu	
435			440			445										
CTG	CTC	AAC	TAC	ATC	GGC	GGC	ACC	ACC	AAC	CGC	GGC	ATC	GTC	AAC	CAG	1390
Leu	Leu	Asn	Tyr	Ile	Gly	Gly	Thr	Thr	Asn	Arg	Gly	Ile	Val	Asn	Gln	
450			455			460										
ACC	ACC	GAG	CAG	CTG	GTG	GAG	CAG	GTG	GAC	AAG	GAC	CTG	CGC	AAC	ATG	1438
Thr	Thr	Glu	Gln	Leu	Val	Glu	Gln	Val	Asp	Lys	Asp	Leu	Arg	Asn	Met	
465			470			475										
GTC	ATC	AAG	CCC	GAC	GCG	CCC	AAG	CCC	CGT	GTG	GTG	GGC	GTG	CGC	GTG	1486
Val	Ile	Lys	Pro	Asp	Ala	Pro	Lys	Pro	Arg	Val	Val	Gly	Val	Arg	Val	
480			485			490			495							
TGG	CCG	CGC	GCC	ATC	CCG	CAG	TTC	AAC	CTG	GGC	CAC	CTG	GAG	CAG	CTG	1534
Trp	Pro	Arg	Ala	Ile	Pro	Gln	Phe	Asn	Leu	Gly	His	Leu	Glu	Gln	Leu	
500			505			510										
GAC	AAG	GCG	CGC	AAG	GCG	CTG	GAC	GCG	GCG	GGG	CTG	CAG	GGC	GTG	CAC	1582
Asp	Lys	Ala	Arg	Lys	Ala	Leu	Asp	Ala	Ala	Gly	Leu	Gln	Gly	Val	His	

	515	520	525	
CTG GGG GGC AAC TAC GTC AGC GGT GTG GCC CTG GGC AAG GTG GTG GAG				1630
Leu Gly Gly Asn Tyr Val Ser Gly Val Ala Leu Gly Lys Val Val Glu				
	530	535	540	
CAC GGC TAC GAG TCC GCA GCC AAC CTG GCC AAG AGC GTG TCC AAG GCC				1678
His Gly Tyr Glu Ser Ala Ala Asn Leu Ala Lys Ser Val Ser Lys Ala				
	545	550	555	
GCA GTC AAG GCC TAA GCGGCTGCAG CAGTAGCAGC AGCAGCATCG GGCTGTAGCT				1733
Ala Val Lys Ala				
560	563			
GGTAAATGCC GCAGTGGCAC CGGCAGCAGC AATTGGCAAG CACTTGGGGC AAGCGGAGTG				1793
GAGGCGAGGG GGGGGCTACC ATTGGCGCTT GCTGGGATGT GTAGT				1838

<210> 11  
 <211> 28  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Designed oligonucleotide primer used for amplifying a DNA fragment containing a *Chlamydomonas reinhardtii*-derived PPO gene

<400> 11  
 AATGATGTTG ACCCAGACTC CTGGGACC 28

<210> 12  
 <211> 27  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Designed oligonucleotide primer used for constructing a vector for expressing a *Chlamydomonas reinhardtii*-derived PPO gene in *Escherichia coli*

<400> 12  
 TACTACACAT CCCAGCAAGC GCCAATG 27

<210> 13  
 <211> 32  
 <212> DNA  
 <213> Artificial Sequence

<220>

<223> Designed oligonucleotide primer used for constructing a vector for expressing a *Chlamydomonas reinhardtii*-derived PPO gene in *Escherichia coli*

<400> 13

TCGAGCTCAA TGATGTTGAC CCAGACTCCT GG 32

<210> 14

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Designed oligonucleotide primer used for constructing a vector for expressing a *Chlamydomonas reinhardtii*-derived PPO gene in *Escherichia coli*

<400> 14

TTGTCGACTA CTACACATCC CAGCAAGCGC CA 32

WHAT IS CLAIMED IS:

1. A method for evaluating the ability of a compound to inhibit the protoporphyrinogen oxidase activity, which comprises the steps of:

(1) culturing a transformant expressing a protoporphyrinogen oxidase gene present in a DNA fragment in a medium containing substantially no protoheme compounds in each comparative system of the presence and absence of a test compound to measure a growth rate of the transformant under each condition, said transformant being resulted from a host cell deficient in the growing ability based on the protoporphyrinogen oxidase activity transformed with the DNA fragment in which a promoter functionable in the host cell and a protoporphyrinogen oxidase gene are operatively linked, and

(2) determining the ability of the compound to inhibit the protoporphyrinogen oxidase activity by comparing the growth rates.

2. A method for evaluating the ability of a compound to inhibit the protoporphyrinogen oxidase activity, which comprises the steps of:

(1) culturing a transformant expressing a protoporphyrinogen oxidase gene present in a DNA fragment in a medium containing substantially no protoheme compounds in each comparative system of the presence and absence of a test compound to measure a growth rate of the transformant under each condition, said transformant being resulted from a host cell deficient in the growing ability based on the protoporphyrinogen oxidase activity transformed with the DNA fragment in which a promoter functionable in the host cell, a protoporphyrinogen oxidase gene and a terminator functionable in the host cell are operatively linked, and



(2) determining the ability of the compound to inhibit the protoporphyrinogen oxidase activity by comparing the growth rates.

3. A method for evaluating the ability of a compound to inhibit the protoporphyrinogen oxidase activity, which comprises the steps of:

(1) culturing a transformant expressing a protoporphyrinogen oxidase gene present in the following (a) DNA fragment in a medium containing substantially no protoheme compounds in each comparative system of the presence or absence of a test compound to measure a growth rate of the transformant under each condition, said transformant being resulted from a host cell deficient in the growing ability based on protoporphyrinogen oxidase activity transformed with

(a) the DNA fragment in which a promoter functionable in the host cell and controllable in its transcriptional activity, and a protoporphyrinogen oxidase gene are operatively linked, and (b) a DNA fragment in which a gene being capable of controlling the transcriptional activity of the promoter in the above DNA fragment and a promoter having the transcriptional activity not controllable by the gene and functionable in the host cell are operatively linked, and

(2) determining the ability of the compound to inhibit the protoporphyrinogen oxidase activity by comparing the growth rates.

4. A method for evaluating the ability of a compound to inhibit the protoporphyrinogen oxidase activity, which comprises the steps of:

(1) culturing a transformant expressing a protoporphyrinogen oxidase gene present in the following (a) DNA fragment in a medium containing substantially no protoheme compounds in each comparative system of the presence and absence of a test

compound to measure a growth rate of the transformant under each condition, said transformant being resulted from a host cell deficient in the growing ability based on the protoporphyrinogen oxidase activity transformed with

(a) the DNA fragment in which a promoter functionable in the host cell and controllable in its transcriptional activity, a protoporphyrinogen oxidase gene and a terminator functionable in the host cell are operatively linked, and

(b) a DNA fragment in which a gene being capable of controlling the transcriptional activity of the promoter in the above DNA fragment, a promoter having the transcriptional activity not controllable by the gene and functionable in the host cell, and a terminator functionable in the host cell are operatively linked, and

(2) determining the ability of the compound to inhibit the protoporphyrinogen oxidase activity by comparing the growth rates.

5. The method according to claim 1 or 3, which is characterized in that the protoporphyrinogen oxidase gene is a protoporphyrinogen oxidase gene derived from an animal or a plant.

6. The method according to claim 1 or 3, which is characterized in that the protoporphyrinogen oxidase gene is a protoporphyrinogen oxidase gene derived from a rat or *Chlamydomonas reinhardtii*.

7 The method according to claim 1 or 3, which is characterized in that the host cell is a microorganism.

8. A rat-derived gene encoding a protein having the protoporphyrinogen oxidase activity.

9. A protoporphyrinogen oxidase gene encoding a protein having the amino acid sequence shown by SEQ ID: No.1.
10. A gene encoding a protein having the protoporphyrinogen oxidase activity and having the amino acid sequence in which one or several amino acids are deleted, substituted, modified or added in the amino acid sequence shown by SEQ ID: No.1.
11. A protoporphyrinogen oxidase gene having the nucleotide sequence encoding the amino acid sequence shown by SEQ ID: No.1.
12. A protoporphyrinogen oxidase gene having the nucleotide sequence shown by SEQ ID: No.2.
13. A DNA fragment having a partial nucleotide sequence of the protoporphyrinogen oxidase gene of any one of claims 8 to 12.
14. A vector which comprises containing the protoporphyrinogen oxidase gene of any one of claims 8 to 12.
15. A transformant which is characterized in that the vector of claim 14 is introduced into a host cell.
16. The transformant according to claim 15, wherein the host cell is a microorganism.
17. The transformant according to claim 15, wherein the host cell is a plant.
18. A *Chlamydomonas reinhardtii*-derived gene encoding a protein having the protoporphyrinogen oxidase activity.
19. A protoporphyrinogen oxidase gene encoding a protein having the amino acid sequence shown by SEQ ID: No.9.

20. A gene encoding a protein having the protoporphyrinogen oxidase activity and having the amino acid sequence in which one or several amino acids are deleted, substituted, modified or added in the amino acid sequence shown by SEQ ID: No.9.
21. A protoporphyrinogen oxidase gene having the nucleotide sequence encoding the amino acid sequence shown by SEQ ID: No.9.
22. A protoporphyrinogen oxidase gene having the nucleotide sequence shown by SEQ ID: No.10.
23. A DNA fragment having a partial nucleotide sequence of the protoporphyrinogen oxidase gene of any one of claims 18 to 22.
24. A vector which comprises the protoporphyrinogen oxidase gene of any one of claims 18 to 22.
25. A transformant which is characterized in that the vector of claim 24 is introduced in a host cell.
26. The transformant according to claim 25, wherein the host cell is a microorganism.
27. The transformant according to claim 25, wherein the host cell is a plant.

## ABSTRACT

The present invention provides:

A method for evaluating the ability of a compound to inhibit the protoporphyrinogen oxidase activity, which comprises the steps of:

(1) culturing a transformant expressing a protoporphyrinogen oxidase gene present in a DNA fragment in a medium containing substantially no protoherne compounds in each comparative system of the presence and absence of a test compound to measure a growth rate of the transformant under each condition, said transformant being resulted from a host cell deficient in the growing ability based on the protoporphyrinogen oxidase activity transformed with the DNA fragment in which a promoter functionable in the host cell and a protoporphyrinogen oxidase gene are operatively linked, and

(2) determining the ability of the compound to inhibit the protoporphyrinogen oxidase activity by comparing the growth rates; and the like.

09289180-040999

Fig.1

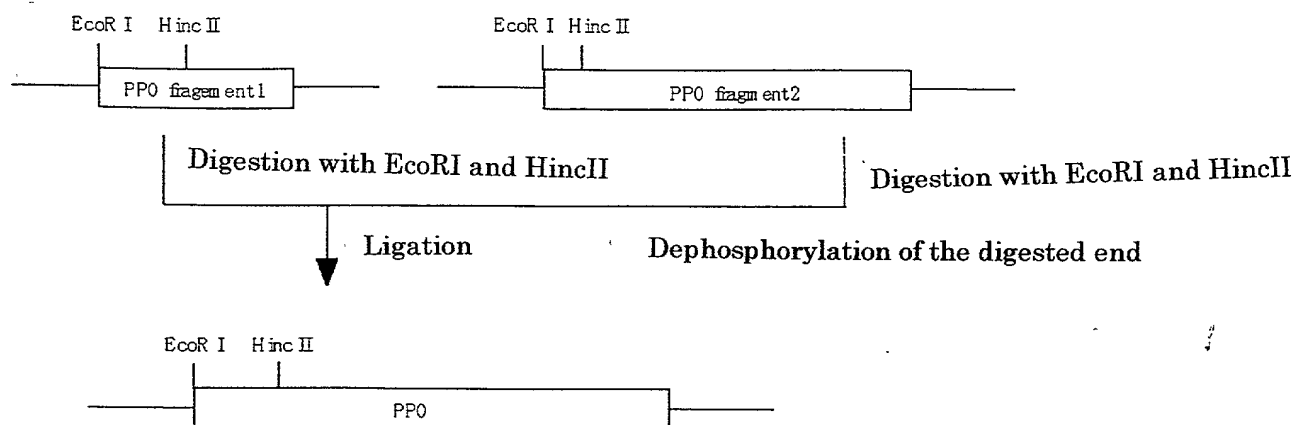


Fig.2

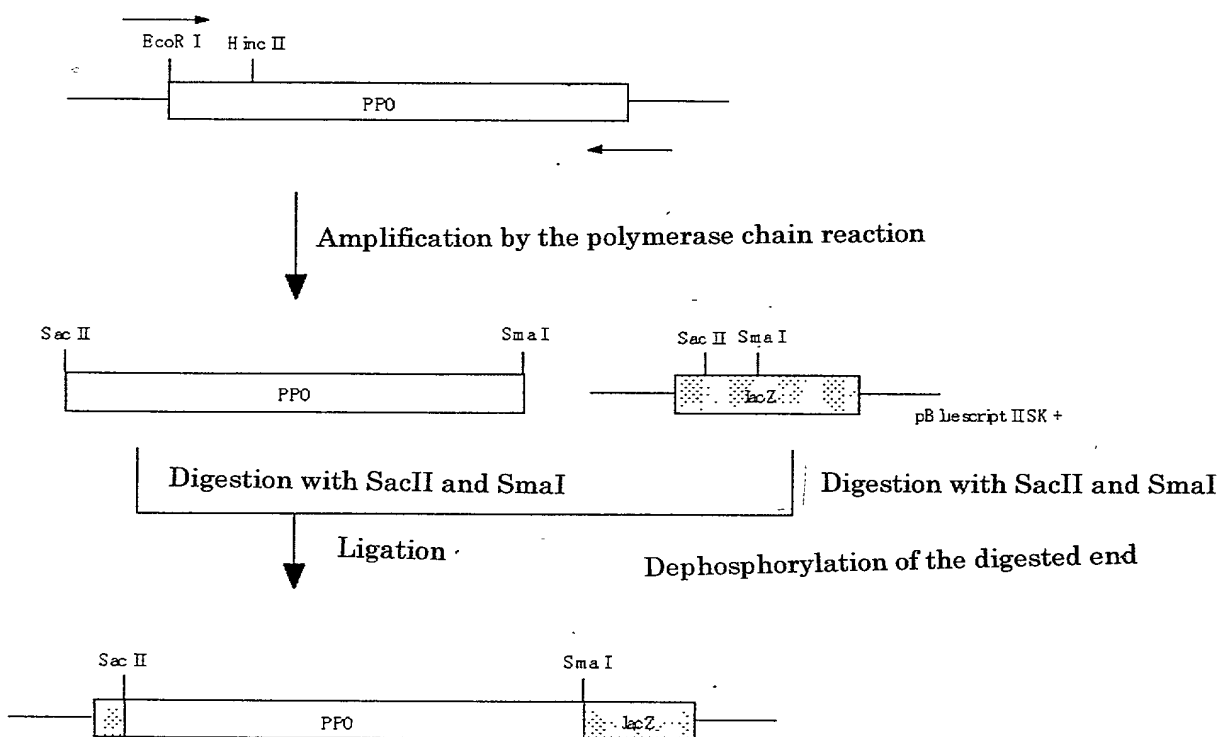


Fig.3

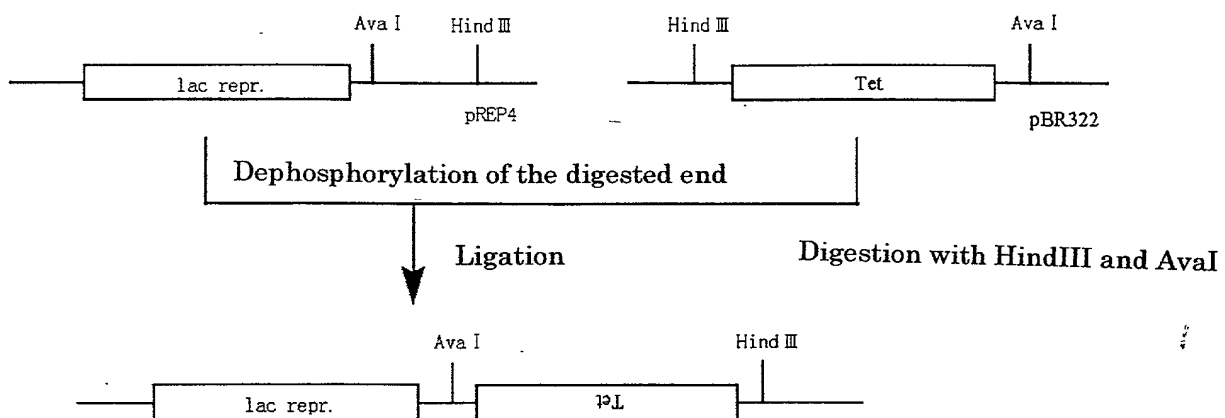


Fig.4

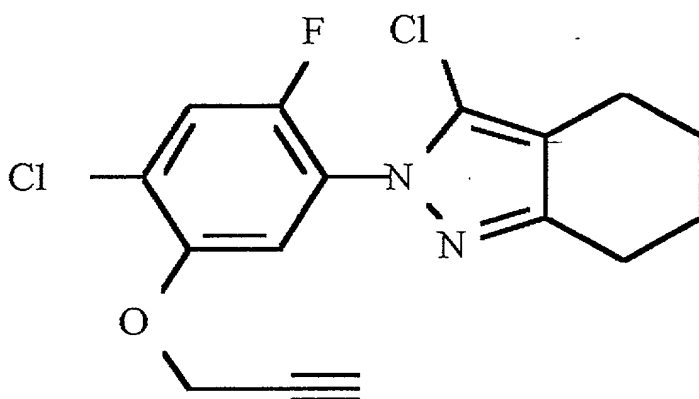


Fig.5

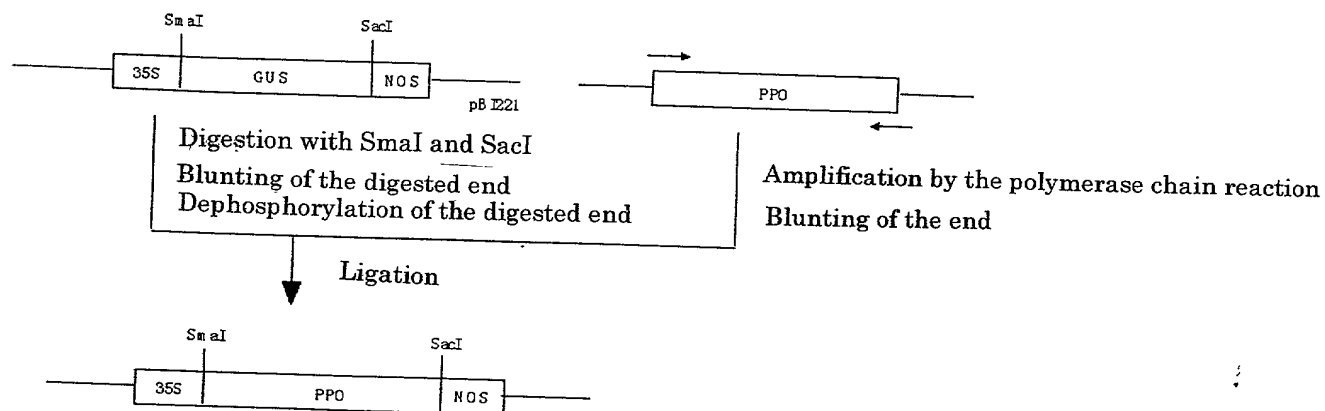


Fig.6

